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ERRATA

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Page 315: In abscissa in Fig. 4 change "10^{-5}" in "10^{-3}".

Page 431: In footnote interchange "ATP" and "ADP".

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Page 19: Improved reproduction of Figs. 1a and 1b. (See overleaf)

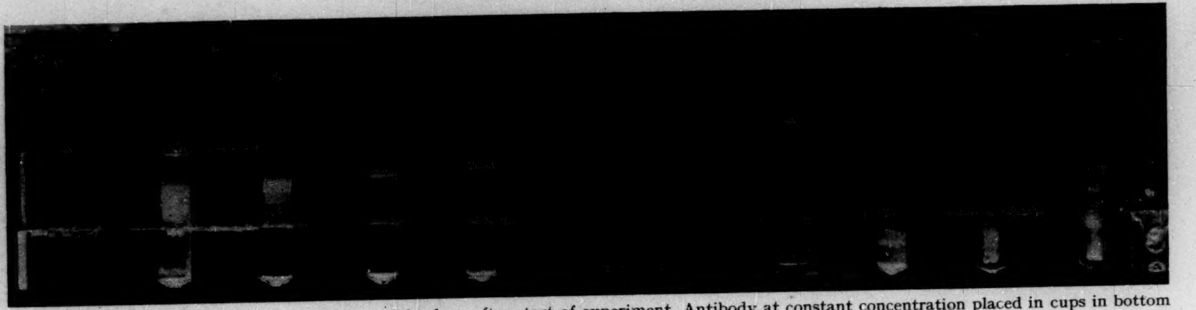

Fig. 1a. Photograph of gel precipitin apparatus eight days after start of experiment. Antibody at constant concentration placed in cups in bottom of apparatus in liquid state, neutral agar layer in central tubes and falling twofold dilutions of antigen in upper cavities. Note minimum band width in region of fourth column.

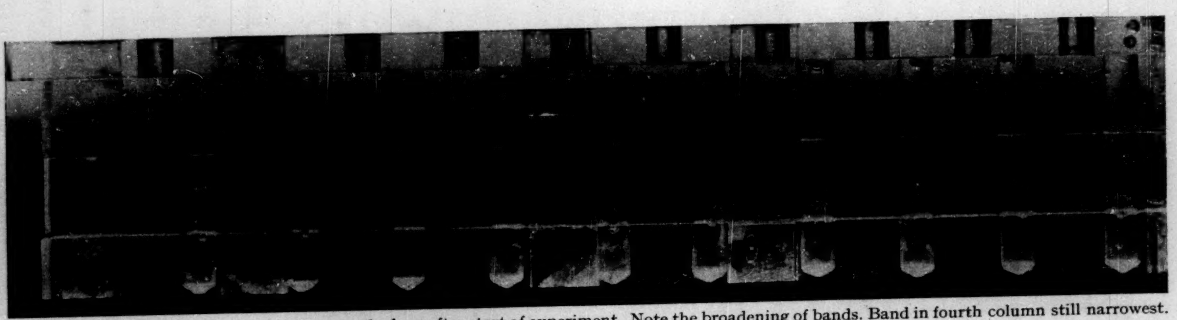

Fig. 1b. Photograph of gel precipitin apparatus 60 days after start of experiment. Note the broadening of bands. Band in fourth column still narrowest. Additional bands, probably artefacts, produced after too long standing.

BIOCHIMICA ET BIOPHYSICA ACTA

ENZYMES OF URACIL METABOLISM IN TISSUES WITH DIFFERENT GROWTH CHARACTERISTICS

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SUMMARY

Different tissues were investigated for their contents of uridine phosphorylase, uridine kinase, deoxyuridine phosphorylase and deoxyuridine kinase activities. These enzyme contents were considered to mirror the relative efficiency of an enzymic pathway using uracil for the biosynthesis of nucleic acid pyrimidines. Furthermore, the same tissues were investigated for their capacities to convert orotic acid to UMP, representing the *de novo* pathway of pyrimidine synthesis. In a survey of some normal tissues from rat, mouse and rabbit, a certain degree of correlation was found between the efficiency of this alternative pathway and the growth rate.

During liver regeneration, marked increases in the enzymes studied were observed. Thus, uridine phosphorylase showed a maximal threefold increase between 36 and 72 h after hepatectomy. Corresponding values were a fivefold increase for uridine kinase (24–36 h), a fourfold increase for deoxyuridine phosphorylase (about 48h), a tenfold increase for deoxyuridine kinase (36–48 h) and, for the enzymes transforming orotic acid to UMP a 5- to 6-fold increase (about 48 h). 144 h after the operation, all enzymes had reverted to almost normal values. These rapid changes in the activities of enzymes involved in the synthesis of nucleotides, might form part of an homeostatic mechanism involved in pyrimidine biosynthesis.

INTRODUCTION

The function of free uracil as an intermediate in polynucleotide synthesis has been the subject of many investigations. Originally PLENTL AND SCHOENHEIMER[1] were unable to find any incorporation of [^{15}N]uracil into rat polynucleotide pyrimidines *in vivo*. Later studies in different laboratories[2–5] with rat tissues *in vivo* and *in vitro* showed a small but definite incorporation of [2-^{14}C]uracil which in most cases, was much smaller than that of orotic acid or pyrimidine nucleosides. With high uracil concentrations, however, CANELLAKIS[6] demonstrated that rat-liver slices utilized this pyrimidine to about the same extent as orotic acid. Even at moderate dose levels, a high incorporation of uracil was observed *in vivo* in rat intestinal mucosa, Flexner-Jobling carci-

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Abbreviations: UMP and dUMP, uridine 5′-monophosphate and deoxyuridine 5′-monophosphate respectively; ADP and ATP, adenosine 5′-di- and -triphosphate respectively; PGA, 3-phosphoglyceric acid; PCA, perchloric acid; Tris, tris(hydroxymethyl)aminomethane; RNA ribosenucleic acid; DNA, deoxyribosenucleic acid; P_i, inorganic phosphate.

noma[7], mouse tissues and, most pronounced, in the Ehrlich ascites tumour[8]. The reactions leading to the synthesis of UMP can be regarded as the first steps in the incorporation of free uracil into polynucleotides[9,10].

In earlier work from this laboratory the following two reactions were shown to be catalyzed by extracts of Ehrlich ascites tumour[11]:

$$\text{Uracil} + \text{ribose-1-phosphate} \xrightarrow[\text{phosphorylase}]{\text{uridine}} \text{uridine} + \text{phosphate}$$

$$\text{Uridine} + \text{ATP} \xrightarrow[\text{kinase}]{\text{uridine}} \text{UMP} + \text{ADP}$$

In spite of the fact that both uridine phosphorylase[12-14] and uridine kinase[12,15] are known to occur in extracts of mammalian liver we could only find a small conversion of uracil to UMP with extracts from rat and mouse liver. From our results[16] it also appeared that in rat liver, uridine kinase activity represented a rate limiting step for UMP synthesis. It could be demonstrated that considerably higher levels of both uridine phosphorylase and kinase occurred in ascites tumour extracts than in rat-liver extracts. This was most pronounced for uridine kinase which, on a protein basis consistently showed 5–30 times higher activity in tumour extract. It thus became clear that the enzyme pattern in the ascites tumour favoured the utilization of uracil for polynucleotide synthesis and it was suggested that this might reflect an adaptation of the tumour to the high requirements of nucleic acid synthesis.

The aim of the present investigation is to assess the relative efficiency of uracil utilization as an alternative pathway for polynucleotide synthesis. For this purpose, the levels of the enzymes transforming uracil to UMP and deoxy-UMP were determined in extracts from different tissues. For comparison, a similar study was made of enzymes transforming orotic acid to UMP. The tissues studied were normal tissues from mouse, rat and rabbit as well as rat liver at different stages of regeneration after partial hepatectomy.

EXPERIMENTAL PROCEDURE

[2-^{14}C]uracil and non-labelled uridine and deoxyuridine were purchased from Schwarz Laboratories, Inc.

[2-^{14}C]deoxyuridine was synthesized enzymically from [2-^{14}C]uracil and non-labelled deoxyuridine by an exchange reaction catalyzed by extracts from *E. coli*[4]. A typical procedure ran as follows: [2-^{14}C]uracil (260 μmoles) and deoxyuridine (260 μmoles) were dissolved in 120 ml of 0.05 *M* Tris buffer pH 7.4. 120 μmoles of phosphate buffer pH 7.4 and 35 ml of *E. coli* extract* corresponding to a total of 1225 mg of wet bacteria were added. Incubation was carried out for 7 h at 37°. Uracil and deoxyuridine were separated by chromatography on starch. After stopping the reaction by heating on a boiling water bath, the incubation mixture was divided in four portions which were chromatographed separately on starch columns (4 cm × 30 cm). Elution was carried out with ammonium acetate–ethanol, pH 9.5. (32 ml of

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* *E. coli* K 12 were obtained through the courtesy of Dr T. Holme, Department of Bacteriology, Karolinska Institutet, Stockholm (Sweden). The bacteria were grown on a synthetic medium and harvested by centrifugation. The cells were disrupted in the press described by Edebo[17] and finally extracted at 0° with 0.0062 *M* phosphate buffer at pH 7.1. Cell debris was removed by centrifugation at 20,000 × *g* for 15 min at 0°.

5 M ammonium acetate, pH 9.5, 352 ml of 95 % ethanol and 112 ml of water). The deoxynucleoside emerged from the column with an R-value of about 1.8 immediately followed by the pyrimidine. After rechromatography in the same system, deoxyuridine essentially free from uracil, was obtained. A complete equilibration of isotope between uracil and deoxyuridine occurred.

[2-^{14}C]uridine was synthesized enzymically by an exchange reaction analogous to the one described above. [2-^{14}C]uracil (90 μmoles) and uridine (100 μmoles) were dissolved in 60 ml of 0.05 M Tris buffer pH 7.4. 60 μmoles of phosphate buffer pH 7.4 and 14 ml of *E. coli* extract corresponding to 490 mg of wet bacteria were added. Incubation for 3 h at 37°. The reaction was stopped by heating on a boiling water bath for 3 min. Separation of uracil and uridine was performed by ion exchange chromatography. The reaction mixture was introduced to a Dowex-2-chloride (200–400 mesh) column 2.5 cm in diameter and 12 cm of length. The column was previously equilibrated with 0.04 M ammonium hydroxide–0.01 M ammonium chloride–0.001 M sodium tetra borate pH 10.2. The pyrimidine was eluted after circa 14 column volumes of 0.2 M ammonium hydroxide–0.05 M ammonium chloride–0.001 M sodium tetra borate, pH 10.0. The column was then washed thoroughly with water and the nucleoside finally eluted with 0.05 M acetic acid. Equilibration of radioactivity between uracil and uridine was obtained during the incubation. The recovery of radioactivity was usually about 90 %.

Ribose-1-phosphate was prepared enzymically as the barium salt with nucleoside phosphorylase from calf spleen[18].

[2-^{14}C]orotic acid was a preparation available in the laboratory. It was originally synthesized according to NYC AND MITCHELL[16].

Preparation of extracts

Most of the experiments were carried out with extracts of acetone powders from the tissues studied. The preparation of these powders and their extraction was described earlier[11,16]. The amount of proteins extracted during this procedure was checked by Kjeldahl analysis.

In the experiments on regenerating rat liver "supernatant fractions" of tissue homogenates were used as described previously[16]. Partial hepatectomy was performed on male albino rats (150–200 g) by removing of the medial and left lateral liver lobes and the posterior lobule of the right lateral lobe.

The Ehrlich ascites tumour was obtained through the courtesy of Prof. G. KLEIN, Department of Tumour Biology, Karolinska Institutet, Stockholm (Sweden).

Determination of enzyme activities

Uridine phosphorylase and uridine kinase: These two enzyme activities were estimated simultaneously by paper chromatographic separation of the radioactive products after enzymic phosphorolysis and phosphorylation of [2-^{14}C]uridine. For this purpose 10.5 μmoles of $MgCl_2$, 2.1 μmoles of ATP, 6.4 μmoles of PGA, 4.3 μmoles of phosphate buffer pH 7.4, 7.5 μmoles of Tris buffer pH 7.4 and [2-^{14}C]uridine (usually 0.45 μmoles) were incubated with tissue extract at 37° for 15 min in a final volume of 0.26 ml. The reaction was stopped by the addition of 0.03 ml of 4 M PCA. The supernatant was centrifugated and heated at 100° for 1 h to break pyrophosphate and purine glycosyl bonds. After cooling on ice, the supernatant was neutralized (indicator paper) with 4 *M* KOH, and $KClO_4$ allowed to precipitate. Aliquots (0.03 ml) and nonlabelled carriers were subjected to descending paper chromatography on Whatman No. 1 filter paper[16]. A complete separation of uracil, uridine and UMP occurred. The different spots were detected by a Mineralight u.v.-lamp, their corresponding areas cut out and eluted with water. Several aliquots from these eluates were plated and counted at infinite thinness in a Tracerlab Sc-18 windowless flow counter. The relative amounts of labelled uracil, UMP and uridine could thus be determined in each run. By applying these values to the original amount of substrate, the amounts of uracil and UMP produced were calculated.

Deoxyuridine phosphorylase and deoxyuridine kinase: The general procedure for the determination of these enzymes was identical with the one described above. However, [2-^{14}C]deoxyuridine was used as substrate and the deproteinized supernatants were not subjected to hydrolysis at 100°. The acid hydrolysis could be omitted because dUMP-pyrophosphates are not formed enzymically[20]. Since the solvent used gave a borderline separation of uracil and deoxyuridine, a blank sample without enzyme was always run parallel and the uracil activity of the blank was subtracted from the sample uracil value*. Due to the low activities of the deoxyuridine kinase the incubation period in most experiments was prolonged to 30 min.

Transformation of orotic acid to uridine phosphates: The formation of labelled uridine phosphates from [2-^{14}C]orotic acid was measured mainly according to HURLBERT AND REICHARD[15]. Thus 3.8 μmoles of ribose-5-phosphate, 3.8 μmoles of ATP, 11.4 μmoles of PGA, 9.5 μmoles of $MgCl_2$, 10.0 μmoles of Tris buffer pH 7.4 [2-^{14}C]orotic acid (usually 0.16 μmoles) and enzyme were incubated for 30 min at 37° in a final volume of 0.42 ml. After stopping the reaction with 0.05 ml of 4 *M* PCA the supernatant was treated after centrifugation in the same way as described above for the uridine enzyme procedure. Orotic acid, UMP and uracil + uridine (formed through dephosphorylation of UMP) were separated on paper with the ammonium acetate–ethanol solvent described above for column chromatography separation of uracil and deoxyuridine. (R_F-values: orotic acid approx. 0.40; UMP approx. 0.14 and uracil + uridine approx. 0.65). The amount of labelled uracil + uridine was always found to be negligible compared to the amount of [2-^{14}C]UMP.

Catabolism of uracil: The enzymic degradation of [2-^{14}C]uracil was measured as described previously[16]. Thus $^{14}CO_2$ from [2-^{14}C]uracil was collected in 4 *M* KOH in the centre well of a Warburg vessel, precipitated as $BaCO_3$ and counted at infinite thickness in an end-window Geiger counter.

RESULTS

Validity of assay methods

pH-optimum of uridine phosphorylase: The pH-optimum of the reaction:

$$\text{Uridine} + P_i \rightleftarrows \text{uracil} + \text{ribose-1-phosphate}$$

was measured in both directions (Fig. 1) using acetone powder extracts from ascites tumour as the enzyme source. For details of experimental procedure see explanation of Fig. 1. The reaction in both directions has a rather broad pH-optimum about pH 8.

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* The blank showed values of 2–3 % of total radioactivity as uracil and less than 0.2 % as dUMP.

A pH-value of 7.4 which was used in the determinations of uridine phosphorylase activities lies quite close to the optimum.

Dependence of uridine phosphorylase and deoxyuridine phosphorylase on phosphate concentration: In Fig. 2 the enzymic phosphorolysis of uridine and deoxyuridine were measured as a function of phosphate concentration. A dialyzed extract from acetone powder of ascites tumour (3 ml of extract dialyzed for 24 h against two 2-l changes of 0.05 M Tris buffer pH 7.4) was used as an enzyme source. Both enzyme activities approach a plateau which was reached at a phosphate concentration of about 0.002 M. In the subsequent determinations of the two phosphorylases, a phosphate concentration of 0.017 M was used.

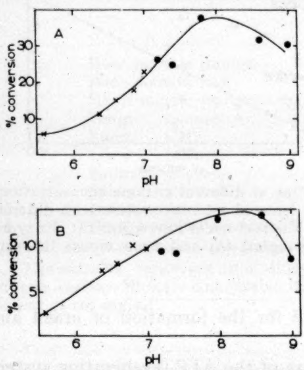

Fig. 1. pH curve of uridine phosphorylase. A. Formation of uridine from uracil and ribose-1-phosphate. Substrate: [2-^{14}C]uracil 1.5 µmole, ribose-1-phosphate 0.8 µmole, buffer (x, imidazole; o, Tris) 50 µmoles. Incubation at 37° 15 min, with 3.0 mg enzyme (acetone powder from ascites tumour). Volume 0.28 ml. B. Phosphorolysis of uridine. Substrate: [2-^{14}C]-uridine, 0.42 µmole, phosphate 10.0 µmoles, buffer 100 µmoles (x, imidazole; o, Tris). Incubation at 37° for 15 min with 3.0 mg enzyme (acetone powder from ascites tumour). Volume 0.23 ml.

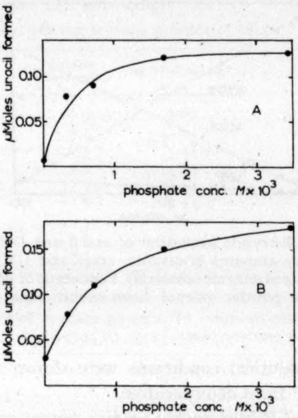

Fig. 2. A. Dependence of uridine phosphorylase on phosphate. Substrate: [2-^{14}C]uridine 0.45 µmole. Tris buffer 10.0 µmoles, pH 7.4, and phosphate as indicated on abscissa. Incubation for 15 min at 37° with 2.2 mg enzyme (dialyzed extract from acetone powder from ascites tumour). Volume 0.26 ml. B. Dependence of deoxyuridine phosphorylase on phosphate. Substrate: [2-^{14}C]deoxyuridine 0.47 µmole. Tris buffer 10.0 µmoles, pH 7.4, and phosphate as indicated on abscissa. Incubation for 30 min at 37° with 7.4 mg enzyme (dialyzed extract from acetone powder from ascites tumour). Volume 0.26 ml.

Enzymic formation of uracil and UMP from uridine: In all determinations of uridine phosphorylase and uridine kinase a wide range of enzyme concentrations was used to ensure the rectilinear portion of the enzyme concentration–activity curve. Fig. 3 shows the formation of uracil and UMP with different amounts of enzyme in two typical experiments. The relative amounts of labelled UMP, uracil and uridine are plotted against the amount of extract used. From the initial slopes of the curves the two enzyme activities were expressed as amounts of uracil or UMP formed per mg of acetone powder during a period of 15 min. In control experiments without enzyme there was no [2-^{14}C]UMP activity although a small amount of [2-^{14}C]uracil was sometimes found which approx. corresponded to 0.2 % of the total radioactivity. The amount of [2-^{14}C]uridine introduced initially was always carefully standardized by u.v. spectrophotometry and gave an initial concentration well above that required for saturation as stated earlier[16].

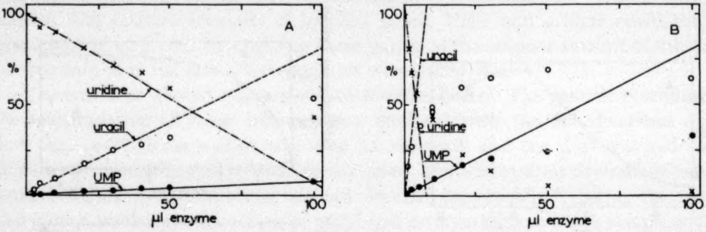

Fig. 3. Enzymic formation of uracil and UMP from uridine at different enzyme concentrations. Relative amounts of uridine, uracil and UMP (ordinate) isolated after incubation with different amounts of enzyme (abscissa). For details of procedure see the text under EXPERIMENTAL. Enzyme: acetone powder extract from rabbit bone marrow 125 mg/ml (A) and from mouse intestine 125 mg/ml (B).

Identical conditions were shown to be valid for the formation of uracil and dUMP from deoxyuridine.

ATP level during uridine kinase assay: Failure of the ATP-regenerating system in the assay mixture would give erroneous low values for uridine kinase activity. In order to check this point, 0.1 ml of the "supernatant fraction" from normal rat liver was incubated with the usual assay system without uridine. The usual procedure was followed but the acid hydrolysis at 100° omitted. After neutralization, the supernatant was chromatographed on a Dowex-2-formate column according to HERBERT *et al.*[22]. In this experiment the presence of 0.1 μmoles of ADP and 1.5 μmoles of ATP could be demonstrated after incubation (2.1 μmoles of ATP were originally added).

Survey of normal tissues from mouse, rat and rabbit

All experiments in this section were carried out with acetone powder extracts from the different tissues.

Uridine phosphorylase and uridine kinase: The results for these two enzymes are summarized in Table I. It is evident that heart muscle, brain and liver contained relatively low levels of both enzymes. The other extreme position was held by mouse intestine and Ehrlich ascites tumours which showed uridine kinase activities 5–15 times greater than those of mouse liver. Mouse intestine gave by far the highest uridine phosphorylase value*. Kidney and spleen occupy an intermediate position with respect to both enzymes and finally bone marrow from rat and rabbit showed values comparable to those of rat liver. These low values were surprising in view of the high rate of cell turnover known to take place in bone marrow and which might indicate that bone marrow depends heavily on *de novo* synthesis of pyrimidines. On the other hand, the preparation of acetone powder from bone marrow was difficult and time consuming and the observed values might be too low.

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* This activity represents a true phosphorolysis of uridine and is not caused by contamination with *e.g.* bacterial hydrolases[21]. Thus, after extensive dialysis the formation of uracil was stimulated more than tenfold by the addition of inorganic phosphate.

TABLE I

DETERMINATIONS OF URIDINE KINASE AND URIDINE PHOSPHORYLASE IN ACETONE POWDER EXTRACTS FROM TISSUES OF MOUSE, RAT AND RABBIT

Activities as mμmoles of uracil or UMP formed per mg acetone powder/15 min.

	Uridine kinase	*Uridine phosphorylase*	*mg N/ml/100 mg**
Bone marrow (rabbit)	2.7	26.9	3.60
Bone marrow (rat)	8.0	5.4	—
Heart muscle (mouse)	3.8	10.3	5.44
Brain (mouse)	9.5	3.0	4.85
Liver (rat)	2.5	14.2	9.33
Liver (mouse)	9.3	12.8	8.25
Kidney (mouse)	12.4	17.1	7.70
Spleen (mouse)	31.0	5.9	7.50
Intestine (mouse)	51.0	346.0	7.26
Ehrlich ascites tumours	50–140	50–140	—

* The extracts were made from different amounts of acetone powder. In order to make the nitrogen analyses directly comparable these values are corrected to an assumed acetone powder content of 100 mg/ml.

TABLE II

DEOXYURIDINE PHOSPHORYLASE, DEOXYURIDINE KINASE AND FORMATION OF UMP FROM OROTIC ACID IN TISSUES OF MOUSE, RAT AND RABBIT

Activities as mμmoles of uracil, dUMP and UMP respectively formed per mg of acetone powder per 15 min.

	Deoxyuridine kinase	*Deoxyuridine phosphorylase*	*UMP from orotic acid*
Bone marrow (rabbit)	0.1	1.3	0.2
Heart muscle (mouse)	0.1	1.2	0.2
Liver (rat)	0.1	5.3	0.2
Kidney (mouse)	0.2	2.1	0.1
Spleen (mouse)	0.3	0.5	1.7
Intestine (mouse)	0.4	90.4	0.2
Ehrlich ascites tumours	1–2	10–30	1–5

Deoxyuridine phosphorylase and deoxyuridine kinase

Table II gives a summary of determinations of these two enzymes carried out with extracts from different tissues. The same general pattern as for uridine phosphorylase and uridine kinase can be recognized. Thus, mouse intestine and Ehrlich ascites tumours gave the highest values for both deoxyuridine phosphorylase and deoxyuridine kinase. Relatively low levels in bone marrow were observed with these enzymes also.

Formation of UMP from orotic acid: These determinations were made as a relative measure of the capacity of the different tissues to utilize orotic acid for nucleic acid synthesis. The transformation of orotic acid to UMP involves only a small part of the *de novo* pathway for the synthesis of pyrimidines. Furthermore, a sum of three enzyme reactions[23,24] is measured simultaneously. If low conversion rates were ascertained, however, the results were reproducible and proportional to enzyme concentrations. The last column of Table II gives determinations of the enzymes in different tissues. It can be seen that a rough parallel existed between the amounts of these enzymes and the phosphorylases and kinases in bone marrow, heart muscle, liver and kidney. Spleen, however, gave a very high activity while intestine showed a rather low value. These results would indicate a preponderance of *de novo* synthesis of pyrimidines *via* orotic acid in spleen, while in the intestine most polynucleotide pyrimidines could be synthesized from preformed uracil, arising *e.g.* from the diet.

Studies on regenerating liver

After hepatectomy the remaining lobe was allowed to regenerate for different periods of time before the animals were killed. These periods were usually multiples of 12 h in order to obtain the enzyme analyses in phase with the diurnal variations of the mitotic index in regenerating rat liver[25]. At each time point chosen, three rats were sacrificed, the liver lobes removed and immediately cooled on ice. Acetone powders were made from one part of the pooled lobes within each group. The pooled rests were homogenized and the "supernatant fractions" were prepared. These supernatants usually showed a nitrogen content of 2.5–3.0 mg N/ml.

Uridine phosphorylase and uridine kinase: Fig. 4 shows uridine phosphorylase and uridine kinase activities in the "supernatant fractions" from regenerating rat liver at different intervals after partial hepatectomy. Both enzymes were measured simultaneously and the two curves of Fig. 4 thus represent the same series of operated animals. The activities are expressed as μmoles of uracil and UMP respectively formed per mg of protein-N during a 15-min period. A sharp rise in uridine phosphorylase occurs after 24 h after the operation with maximal activities between 36 and 72 h. After that, enzyme activity proceeded to decrease slowly and at the 144-h time

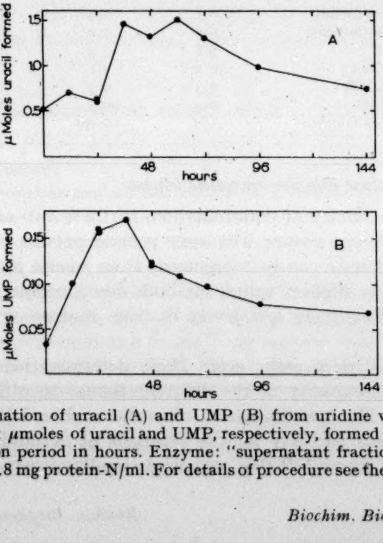

Fig. 4. Enzymic formation of uracil (A) and UMP (B) from uridine with extracts from regenerating liver. Ordinate: μmoles of uracil and UMP, respectively, formed per mg of protein nitrogen. Abscissa: regeneration period in hours. Enzyme: "supernatant fractions" from regenerating rat liver containing 2.3–2.8 mg protein-N/ml. For details of procedure see the text under EXPERIMENTAL.

point the activity was almost back to normal. Fig. 4B gives the corresponding curve for uridine kinase. The peak activity of the enzyme was reached earlier than that of phosphorylase. Thus, a rise had already begun 12 h after surgery. Maximal activities were measured at 24 and 36 h,followed by a steady decline. It should be noted that, at every time point uridine phosphorylase exhibited activities which were about ten times higher than those of uridine kinase.

Deoxyuridine phosphorylase and deoxyuridine kinase: These enzymes were measured in extracts of acetone powders from regenerating rat liver. This allowed experiments with much more concentrated protein solutions than was possible with "supernatant fractions". The procedure was adopted because of the small quantities of these activities present in liver. Fig. 5A shows deoxyuridine phosphorylase activities. At 48 h after surgery, a rise, about four times greater than the activity curve of nonregenerating liver can be seen. At 144 h, the activity had almost reverted to that of normal liver. A similar curve was obtained for deoxyuridine kinase (Fig. 5B). This enzyme showed the most marked rise in activity of all the enzymes studied. The maximum was reached after 36 h and represented a tenfold rise over normal liver. Deoxyuridine phosphorylase activity was 10–20 times greater than that of deoxyuridine kinase throughout the whole regeneration period.

Formation of UMP from orotic acid: These determinations were also performed with acetone powder extracts. Fig. 6 shows a sharp peak in enzyme activity at 48 h after hepatectomy. The activity then drops sharply and has reached almost normal values 70–80 h after the operation.

Catabolism of uracil: From the "supernatant fractions" used for the analyses of uridine phosphorylase and uridine kinase, samples were withdrawn and immediately tested for their ability to decompose uracil to CO_2 as described earlier[16]. As can be seen from Fig. 7, the capacity of liver to decompose uracil began declining after 24 h.

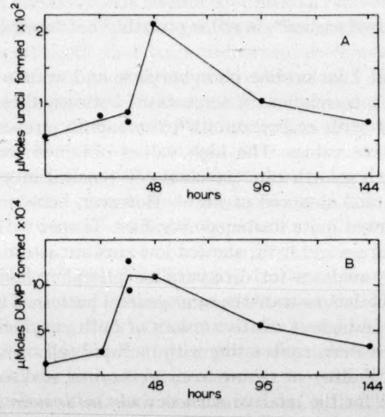

Fig. 5. Enzymic formation of uracil (A) and dUMP (B) from deoxyuridine with extracts from regenerating liver. Ordinate: μmoles of uracil and dUMP, respectively, formed per mg of acetone powder. Abscissa: regeneration period in hours. Enzyme: acetone powder extracts from regenerating liver 166 mg/ml. For details of procedure see the text under EXPERIMENTAL.

A minimum is reached after 48–60 h, after which time a slow rise takes place. Normal values were again obtained after 144 h. This curve is a confirmation of the earlier results of CANELLAKIS[26].

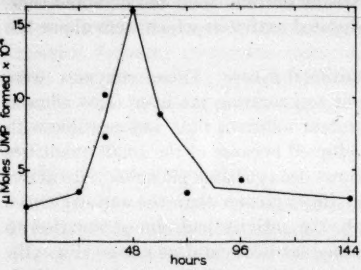

Fig. 6. Enzymic formation of UMP from orotic acid. Ordinate: μmoles of UMP formed/mg acetone powder. Abscissa: regeneration period in hours. Enzyme: acetone powder extracts from regenerating liver, 166 mg/ml.

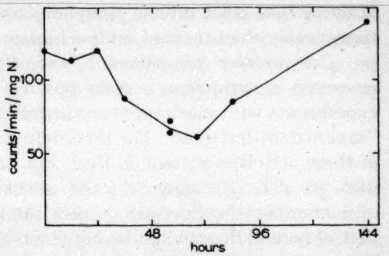

Fig. 7. Enzymic formation of $^{14}CO_2$ from [2-^{14}C]uracil with extracts from regenerating liver. Conditions as described under EXPERIMENTAL. Ordinate: counts/min ($BaCO_3$ at infinite thickness) per mg protein nitrogen in extract. Abscissa: regeneration period in hours. Each point is calculated from three experiments performed with 0.3 ml 0.5 ml and 1.0 ml respectively of "supernatant fraction" from regenerating liver (2.3–2.8 mg protein nitrogen/ml).

DISCUSSION

The present investigation was undertaken with the primary aim of investigating whether tissues with different growth characteristics showed variations in their ability to utilize uracil for nucleotide synthesis. Such an alternative pathway, which utilizes the combined action of nucleoside phosphorylases and kinases, might possibly be regarded as a "reserve mechanism" for nucleic acid synthesis in tissues with rapid growth rates. As discussed earlier[16], uracil is probably not formed *de novo* from small molecules.

The values of Table I for uridine phosphorylase and uridine kinase activities in different tissues show a correlation in some cases between these enzymes and the growth rate. Consistent with earlier results[16], the rapidly growing ascites tumours gave the highest relative values. The high values obtained for intestine are also compatible with its high growth rate (mucosal cell renewal in 35 h[27]) and the good incorporation of free uracil observed earlier[2,7]. However, bone marrow with its very rapid cell turnover showed quite low enzyme values. Tissues with low growth rates, such as heart muscle, brain and liver, showed low enzyme activities.

The corresponding analyses for deoxyuridine phosphorylase and deoxyuridine kinase shown in Table II demonstrate the same general pattern. Thus, ascites tumours and intestine showed the highest relative values of both enzymes. In this case, too, bone marrow gave low values, contrasting with its rapid cell turnover.

The capacities of the different tissues to convert orotic acid to UMP were investigated as representative for the relative efficiency of the *de novo* synthesis of nucleic acid pyrimidines. If these conversion capacities (Table II) are compared to the general pattern of phosphorylase and kinase activities, two striking differences emerge. Intestine gives a very low value and spleen a relatively high one, compared to the relative concentrations of phosphorylases and kinases in these tissues. These data might reflect a strong domination of the uracil utilizing pathway in intestine and of the orotic acid pathway in spleen.

Regenerating liver has a very rapid growth rate, it is capable of doubling its weight in about 48 h[28]. In correlation with this fact, the enzymes leading to the synthesis of UMP and dUMP showed greatly elevated tissue concentrations during liver regeneration. All the enzymes studied reverted to levels close to those of normal liver when the regeneration was complete. Uridine kinase showed an earlier and more distinct peak than uridine phosphorylase. The maximum was reached between 24 and 36 h after surgery compared to between 36 and 72 h for uridine phosphorylase. Moreover the total increase is 5.3 fold as compared to 3 fold for uridine phosphorylase. The maximum for the kinase almost coincides with those for RNA-pyrimidine turnover[29] and amino acid incorporation[30] observed earlier in regenerating liver, which both occurred 30 h after operation. However, the main increase in uridine phosphorylase activity occurred definitely after those events. Thus, after 24 h, no certain increase had occurred, even though at that time RNA-pyrimidine synthesis is far above normal, as measured by the incorporation of different precursors. A picture similar to that of uridine phosphorylase was obtained for the enzymes involved in the transformation of orotic acid to UMP and for deoxyuridine phosphorylase and kinase. All these enzymes showed rather late maxima. The maxima for the latter two enzymes should be compared with the maximum of DNA-synthesis, which occurs at 24 h[29]. This time lag, however, is in agreement with the observations of BOLLUM AND POTTER[31] that thymidine incorporating enzymes in regenerating liver continued to increase while DNA-synthesis *in vivo* was decreasing. The total increase in thymidine incorporating enzymes was by factors of 10–25 or more over normal nonregenerating liver. The observed tenfold increase in deoxyuridine kinase in the present experiments is consistent with the pronounced increase in thymidine kinase observed by CANELLAKIS[26]. It seems quite likely that the phosphorylation of thymidine and deoxyuridine is carried out by the same enzyme.

It should be pointed out that both uridine and deoxyuridine kinases in regenerating liver in general have an activity 10–20 times lower than the corresponding phosphorylases. This, together with their earlier increase during regeneration, might reflect their rate-limiting character in the utilization of uracil for the synthesis of nucleic acid pyrimidines.

The determinations of uracil catabolism during liver regeneration reproduce rather closely the results obtained earlier by CANELLAKIS *et al.*[26]. Thus, a 50% decrease was found between 48–60 h after hepatectomy. CANELLAKIS[6,26] has suggested that the increased incorporation of uracil into RNA during liver regeneration might be due to the diminished catabolism of uracil. In the present investigation, evidence is given for greatly increased tissue concentrations of the enzymes involved in the anabolism of uracil during liver regeneration. It is probable that the homeostatic mechanism involved in pyrimidine synthesis is dependent on both the decrease of uracil catabolism and the increase in the capacity to anabolize uracil.

All determinations in this work were carried out with cell extracts. The information was obtained from experiments which aimed at the elucidation of enzyme activities at optimal conditions of substrate concentrations. These conditions do not necessarily prevail to the same extent in all cells studied. Such considerations make it difficult to use the information obtained in the present experiments for conclusions concerning the relative efficiencies of the orotic acid pathway and uracil pathway *in vivo* in different tissues. It is nevertheless considered that approximative comparisons between different cells can be made as far as the same enzyme, or possibly enzyme sequence is concerned. These considerations of relative efficiencies of metabolic routes for the synthesis of nucleic acid pyrimidines in different tissues might be of some import to the discussion on the mechanisms of growth inhibition by pyrimidine analogues.

ACKNOWLEDGEMENT

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REFERENCES

[1] A. A. Plentl and R. J. Schoenheimer, *J. Biol. Chem.*, 153 (1944) 203.

[2] K. C. Leibman and C. Heidelberger, *Federation Proc.*, 14 (1955) 243.

[3] E. S. Canellakis, *Federation Proc.*, 14 (1955) 324.

[4] P. Reichard, *Acta Chem. Scand.*, 9 (1955) 1275.

[5] A. Cantarow, T. L. Williams and K. E. Paschkis, *Federation Proc.*, 15 (1956) 30.

[6] E. S. Canellakis, *J. Biol. Chem.*, 227 (1957) 701.

[7] C. Heidelberger, K. C. Leibman, Eberhard Harbers and P. M. Bhargava, *Cancer Research*, 17 (1957) 399.

[8] U. Lagerkvist, P. Reichard, B. Carlsson and J. Grabosz, *Cancer Research*, 15 (1955) 164.

[9] R. B. Hurlbert and V. R. Potter, *J. Biol. Chem.*, 209 (1954) 1.

[10] S. Ochoa, *Federation Proc.*, 15 (1956) 832.

[11] P. Reichard and O. Sköld, *Acta Chem. Scand.*, 11 (1957) 17.

[12] E. S. Canellakis, *J. Biol. Chem.*, 227 (1957) 329.

[13] C. E. Cardini, A. C. Paladini, R. Caputto and L. F. Leloir, *Acta Physiol. Latinoam.*, 1 (1950) 57.

[14] M. Friedkin and I. Roberts, *J. Biol. Chem.*, 207 (1954) 257.

[15] R. B. Hurlbert and P. Reichard, *Acta Chem. Scand.*, 9 (1955) 251.

[16] P. Reichard and O. Sköld, *Biochim. Biophys. Acta*, 28 (1958) 376.

[17] L. Edebo, *J. Biochem. Microbiol. Technol. and Engineering*, in the press.

[18] V. E. Price ,M. C. Otey and P. Plesner, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. II. Academic Press Inc., Publishers, New York 1955, p. 448.

[19] J. F. Nyc and H. K. Mitchell, *J. Am. Chem. Soc.*, 69 (1947) 1382.

[20] M. Friedkin and A. Kornberg, in W. D. McElroy and B. Glass, *The Chemical basis of heredity*, Johns Hopkins Press, Baltimore, 1957, p. 609.

[21] Y. Takagi and B. L. Horecker, *J. Biol. Chem.*, 225 (1957) 77.

[22] E. Herbert, V. R. Potter and Y. Takagi, *J. Biol. Chem.*, 213 (1955) 923.

[23] A. Kornberg, I. Liebermann and E. S. Simms, *J. Biol. Chem.*, 215 (1955) 389.

[24] I. Liebermann, A. Kornberg and E. S. Simms, *J. Biol. Chem.*, 215 (1955) 403.

[25] J. J. Jaffe, *Anat. Record*, 120 (1954) 935.

[26] E. S. Canellakis, J. J. Jaffe, R. Mantsavinos and J. S. Krakow, *J. Biol. Chem.*, 234 (1959) 2096.

[27] C. P. Leblond and C. E. Stevens, *Anat. Record*, 100 (1948) 357.

[28] G. M. Higgins and R. M. Anderson, *Arch. Pathol.*, 12 (1931) 186.

[29] N. A. Eliasson, E. Hammarsten, P. Reichard, S. Åqvist, B. Thorell and G. Ehrensvärd, *Acta Chem. Scand.*, 5 (1951) 431.

[30] A. von der Decken and T. Hultin, *Exptl. Cell Research*, 13 (1957) 83.

[31] F. J. Bollum and V. R. Potter, *Cancer Research*, 19 (1959) 561.

ION-EXCHANGE CHROMATOGRAPHIC INVESTIGATION OF THE NUCLEOTIDE CONTENT OF LIVER AND SKELETAL MUSCLE OF GUINEA PIGS TREATED BY FEVER-PRODUCING SUBSTANCES

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SUMMARY

The compcsition of several nucleotides in both liver and skeletal muscle has been studied by ion-exchange chromatography in normal guinea pigs, as well as in guinea pigs treated either with 2,4-dinitrophenol or with the lipopolysaccharide of *Salmonella abortus equi*. A decrease in the fraction containing ATP and ADX (an unknown adenosine compound) has been recorded in both tissues of the treated animals. This change also occurred in treated animals which were anaesthetized by magnesium sulfate 15 min before death.

INTRODUCTION

The hypothesis that the increased production of heat occurring during fever is related to the decreased efficiency of oxidative phosphorylation was first put forward by LARDY AND ELVEHJEM[1]. This hypothesis was based on the fact that well-known fever-producing substances, such as 2,4-dinitrophenol (DNP)* inhibit oxidative phosphorylation and stimulate adenosine triphosphatase *in vitro*. The hypothesis was also supported by the results of RONZONI AND EHRENFEST[2] and of LARDY AND PHILLIPS[3], who described a decrease of phosphocreatine, as well as an increase of glycolysis, in the tissues of animals treated by DNP. Recently, PASQUINELLI AND CALZOLARI[4] and PASQUINELLI, CALZOLARI AND D'ALESSANDRO[5] have shown that the phosphorylation of injected glucose is decreased in rabbits treated with a fever-producing yeast hydrolysate. ATPase activity increases in both the liver and skeletal muscle. DIANZANI AND SCURO[6] and DIANZANI AND DIANZANI MOR[7] have found a decreased P/O ratio in some tissues of rats treated by DNP. ATPase activity of mitochondria was found to be increased. Similar results were obtained by VORBRODT[8] and by ALDRIDGE AND STONER[9]. The latter authors used 3,5-dinitro-orthocresol in their investigations. ATPase activity of mitochondria increases also after treatment with other fever-producing substances, such as the lipopolysaccharide of *Salmonella abortus equi* (LPS) and lysergic acid diethylamide[10]. The mitochondria contained in the tissues of animals treated with these substances appear to be considerably swollen[11].

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* The following abbreviations were used throughout this paper: DNP, 2,4-dinitrophenol; LPS, lipopolysaccharide of *Salmonella abortus equi*; PCA, perchloric acid; DPN, diphosphopyridine nucleotide; AMP, adenosine monophosphoric acid; TPN, triphosphopyridine nucleotide; IMP, inosine monophosphoric acid; ADP, adenosine diphosphoric acid; FAD, flavine adenosine dinucleotide; GDP, guanosine diphosphoric acid; ATP, adenosine triphosphoric acid; ITP, inosine triphosphoric acid.

The acid-labile phosphorus content of the tissues of rats treated with 3,5-dinitro-*o*-cresol, another fever-producing substance structurally related to DNP, was found by STONER, THRELFALL AND GREEN[12] to be decreased.

Similar results were obtained also by PARKER[13] in rats treated with lethal amounts of this substance. The extent of the decrease was higher in rats which survived for a longer time. A decrease in the acid-labile phosphorus, as well as in the phosphocreatine, has been found also by DIANZANI[14] in several organs of guinea pigs treated either with DNP or with LPS. The decrease of ATP in the liver of animals treated by DNP was shown also by an enzymic method of determination.

The present paper describes the results of an investigation on the nucleotide content of both liver and skeletal muscle of guinea pigs, as detected by the ion-exchange chromatographic method of SIEKEVITZ AND POTTER[15]. The determination was made in normal animals, as well as in guinea pigs previously injected with DNP or with LPS.

MATERIALS AND METHODS

Guinea pigs weighing 300–350 g, fed on a vegetable diet were used. The temperature of the room in which they lived was 22 ± 2°. The thermal stability of the animals was checked for 7–10 days before the treatment. Colon temperature measurements were made with an electric apparatus (Hartmann and Braun).

DNP was given intraperitoneally in amounts of 4 mg/100 g of body weight, as the sodium salt (Merck). LPS was administered in a similar way, in amounts of 0.05 μg/100 g of body weight. This was kindly supplied by the firm of Wander of Bern.

The animals were killed by decapitation. Those which had been previously injected with the fever-producing substances were killed 2 h after the injection, when the body temperature had increased by about 2°. Immediately after the removal of the head, the liver (about 13 g) and the muscles of the legs (also about 13 g) were dissected in a cold room at 2° and placed in a small blendor (MSE type) containing 26 ml of 1.5 *M* perchloric acid (PCA). The weight of the homogenizer containing the PCA had previously been determined. The exact weight of the amount of tissue used was calculated by weighing the container again. The time between the death of the animal and the immersion of the tissue in PCA was 16–20 sec in the case of the liver, 50–70 sec in that of the muscle. The homogenization was started within 2 min after the death of the animals in both cases.

The homogenates were then centrifuged at 1000 *g* for 10 min. The sediment was resuspended in 26 ml of 0.6 *M* PCA, homogenized and submitted to a new centrifugation at the same speed for the same time. The combined supernatant fluids were cooled in chopped ice and neutralized with 1 *N* KOH. The neutralized fluid was then placed onto the chromatographic column. This was 15 cm high and 1 cm wide. It was filled with Dowex 1-X4 resin. The chloride form of this had been previously converted into the formate form by washing with formic acid. Other washings were made with deionized water until the acid reaction disappeared.

Elution of the nucleotides from the column was carried out with the formic acid–

ammonium formate procedure described by SIEKEVITZ AND POTTER[15]. The eluates were collected in a Shandon fraction collector.

The optical density of each fraction was read at 260 mμ in a Beckman spectrophotometer Mod. D.U., after convenient dilution.

The values of the optical density were taken as a measure of the concentration of the nucleotides. The average values ± the standard deviation are reported in Table I.

TABLE I

NUCLEOTIDE CONTENT IN LIVER AND SKELETAL MUSCLE OF NORMAL GUINEA PIGS, AND OF GUINEA PIGS TREATED EITHER WITH DNP OR WITH LPS

The data represent the optical density at 260 mμ of nucleotides corresponding to 1 g of fresh tissue. The values whose difference from the normal is statistically significant ("P" < 0.05) are indicated with *. A factor of 1000 was used in representing the data.

Fraction	*Normal*	*Anaesthetized by* $MgSO_4$	*Treated with DNP*	*Treated with LPS*
A. Liver				
DPN	366 ± 19	400 ± 20	422 ± 146	371 ± 168
AMP	2364 ± 540	1295 ± 212	1998 ± 665	1952 ± 436
TPN	242 ± 172	96 ± 54	137 ± 99	171 ± 97
IMP	409 ± 67	256 ± 212	507 ± 180	498 ± 77
"AD"	496 ± 127	682 ± 210	607 ± 105	566 ± 115
ADP	959 ± 249	1494 ± 227	1128 ± 237	1011 ± 130
FAD	152 ± 34	128 ± 58	212 ± 149	205 ± 191
GDP	300 ± 112	313 ± 53	274 ± 135	404 ± 143
ATP–ADX	1733 ± 184	2332 ± 162*	1365 ± 75*	1315 ± 161*
ITP	111 ± 29	234 ± 5*	105 ± 36	105 ± 49
B. Muscle				
DPN	72 ± 37	117 ± 13	78 ± 7	152 ± 51
AMP	615 ± 36	730 ± 101	711 ± 143	663 ± 314
TPN	200 ± 80	92 ± 10	257 ± 65	255 ± 59
IMP	175 ± 57	120 ± 77	238 ± 55	66 ± 8*
"AD"	638 ± 114	272 ± 104	1118 ± 299*	666 ± 102
ADP	1836 ± 245	1041 ± 195*	1498 ± 220	1294 ± 357
FAD	333 ± 136	138 ± 87	76 ± 38*	200 ± 27
GDP	173 ± 110	143 ± 116	175 ± 45	171 ± 110
ATP–ADX	5934 ± 162	6284 ± 891	3176 ± 458*	5494 ± 55*
ITP	221 ± 34	159 ± 132	61 ± 21*	126 ± 14*

The significance of the differences between two averages was estimated by calculating the "t" value of FISHER. Only the differences with a "t" value corresponding to a probability $P < 0.05$ were retained as significant.

In some experiments, the animals were anaesthetized by intraperitoneal injection of magnesium sulfate, according to the method of DUBOIS, ALBAUM AND POTTER[16], 15 min before to be killed. This treatment was applied in order to decrease the dephosphorylation of ATP probably occurring during the time between the death of the animal and homogenization.

RESULTS

The results obtained are shown in the figures. Their statistical evaluation is given in Table I. In the figures, the number of the tube, containing a fractions of 10 g of the eluate, is reported under the abscissa, while the optical density is given under the ordinata. The values are referred to 13 g of fresh tissue and represent the average of at least 5 determinations.

Thirteen absorption peaks were observed for both liver and skeletal muscle. They are indicated on the diagrams with arabic numerals. The identification of the substances corresponding to each peak was made by comparison with a chromatogram run of known substances. It was possible to identify in this way only 9 substances: peak No. 3 was due to diphosphopyridine nucleotide; peak No. 4 to adenosine monophosphate; peak No. 5 to triphosphopyridine nucleotide; peak No. 6 to inosine monophosphate; peak No. 8 to adenosine diphosphate; peak No. 9 to flavine adenyldinucleotide; peak No. 10 to guanosine diphosphate; peak No. 12 to adenosine triphosphate and peak No. 13 to inosine triphosphate. According to SIEKEVITZ AND POTTER[15], peak No. 9 contains, in addition to flavine adenyldinucleotide, also an unidentified uridine compound (UDX); peak No. 11, which is indicated by these authors as corresponding to an unidentified adenosine compound (ADX), was not

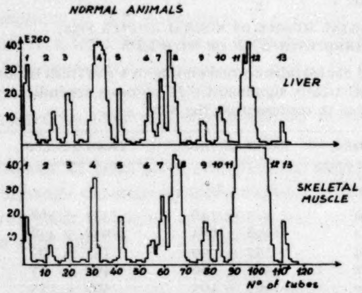

Fig. 1. Nucleotide content of both liver and skeletal muscle of normal guinea pigs.

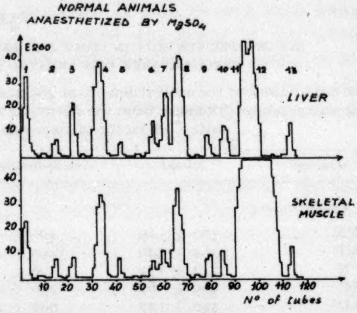

Fig. 2. Nucleotide content of both liver and skeletal muscle of normal guinea pigs anaesthetized by $MgSO_4$.

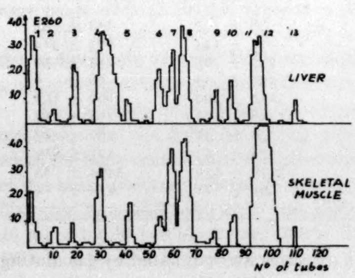

Fig. 3. Nucleotide content of both liver and skeletal muscle of guinea pigs treated with DNP.

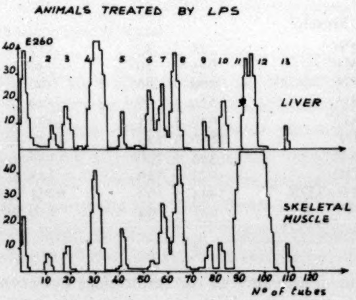

Fig. 4. Nucleotide content of both liver and skeletal muscle of guinea pigs treated with LPS.

completely separable from adenosine triphosphate. The substances responsible for peaks Nos. 1, 2 and 7 were not identified. According to SIEKEVITZ AND POTTER[15], the last peak corresponds to an unknown adenosine compound, designated as "AD".

The heights of the peaks show some differences between the liver and skeletal muscle. Peaks Nos. 1, 3, 4 and 9 are taller for the liver than for the skeletal muscle. On the contrary, peaks No. 8, 11, 12 and 13 are taller for the latter tissue.

In normal animals treated with $MgSO_4$, the amount of ADX + ATP, as well as that of ITP, present in the liver was slightly higher than in untreated guinea pigs. No difference was found between anaesthetized and unanaesthetized animals in the case of skeletal muscle.

In guinea pigs treated with 2,4-dinitrophenol the only fraction which was found significantly different from the normal in both liver and muscle was that containing ADX + ATP, which was strongly decreased. The extent of the decrease was higher in the latter tissue. In skeletal muscle significant decreases of FAD and of ITP were also observed, as well as an increase in the height of peak No. 7 ("AD"). Very similar results were obtained in guinea pigs treated with DNP followed by anaesthetization with $MgSO_4$. A large decrease of the fraction containing ADX + ATP was recorded also in the animals treated with LPS. The decrease was significant in both liver and skeletal muscle, but its extent was less than that observed in the case of DNP-treated guinea pigs.

In the latter, ITP was found to be decreased in the muscle, but remained unchanged in the liver. As opposed to the guinea pigs treated with DNP, IMP was found to be decreased in skeletal muscle, while FAD + UDX and "AD" remained unchanged.

The results obtained with guinea pigs anaesthetized with $MgSO_4$ did not differ from those observed in unaesthetized animals, also in the case of LPS treatment.

DISCUSSION

Parenteral treatment with LPS or with DNP produces a noticeable decrease in the nucleotide fraction containing ADX + ATP in both liver and skeletal muscle of guinea pigs. As these components were not separated from each other, it is impossible to know with certainty if both, or only one of them, is responsible for the decreased height of the peak.

A decrease of ATP in both liver and skeletal muscle has been found by DIANZANI[14] by an enzymic method of determination. It seems probable then that the results obtained in this paper may be interpreted in the sense that the ATP concentration is decreased. In the skeletal muscle, another triphosphate (ITP) is also decreased.

The fact that the DPN concentration remains unchanged agrees with previous results obtained by another method of determination[17]. It seems possible that the cause for the decrease in the height of peaks Nos. 11, 12 and 13 is related to the impairment of oxidative phosphorylation, as well as to the activation of ATPase[5-10].

It seems, however, improbable that this decrease is connected with an eventual increase of preagonic contractions. In fact, a decrease of ATP was also recorded in guinea pigs anaesthetized by $MgSO_4$, whose muscles were relaxed. The fact that no change in the ATPase activity of myofibrils occurs in guinea pigs treated either by DNP or by LPS[10] favours this interpretation.

REFERENCES

[1] H. A. LARDY AND C. A. ELVEHJEM, *Ann. Rev. Biochem.*, 14 (1945) 1.
[2] E. RONZONI AND E. EHRENFEST, *J. Biol. Chem.*, 115 (1936) 749.
[3] H. A. LARDY AND P. H. PHILLIPS, *J. Biol. Chem.*, 149 (1943) 177.
[4] F. PASQUINELLI AND G. CALZOLARI, *Arch. sci. biol.* (*Italy*), 37 (1953) 507.
[5] E. PASQUINELLI, G. CALZOLARI AND A. D'ALESSANDRO, *Giorn. biochim.*, 3 (1954) 359.
[6] M. U. DIANZANI AND S. SCURO, *Biochem. J.*, 62 (1956) 205.
[7] M. U. DIANZANI AND M. A. DIANZANI MOR, *Nature*, 179 (1957) 522.
[8] A. VORBRODT, *Exptl. Cell Research*, 12 (1957) 154.
[9] W. N. ALDRIDGE AND H. B. STONER, *Biochem. J.*, 74 (1960) 148.
[10] M. A. DIANZANI MOR AND M. U. DIANZANI, *Boll. soc. ital. biol. sper.*, 35 (1959) 511.
[11] A. NOVELLI, personal communication.
[12] H. B. STONER, C. J. THRELFALL AND H. N. GREEN, *Brit. J. Exptl. Pathol.*, 33 (1952) 398.
[13] V. PARKER, *Biochem. J.*, 57 (1954) 381.
[14] M. U. DIANZANI, data reported by L. MICHELAZZI AND M. U. DIANZANI, *Atti congr. soc. ital. patol.* 6 (1959) 1.
[15] P. SIEKEVITZ AND V. R. POTTER, *J. Biol. Chem.*, 215 (1955) 221.
[16] K. P. DUBOIS, H. G. ALBAUM AND V. R. POTTER, *J. Biol. Chem.*, 147 (1943) 699.
[17] M. A. DIANZANI MOR, *Giorn. biochim.*, 7 (1959) 215.

DETERMINATION OF THE DIFFUSION CONSTANT OF POLIOVIRUS BY THE GEL PRECIPITIN TECHNIQUE

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SUMMARY

A gel diffusion technique is recommended for the determination of the diffusion constants of materials of which only small quantities are available. In the present work this technique has been applied to the investigation of the diffusion rate of a type II poliovirus strain. A diffusion constant of $1.29 \cdot 10^{-7}$ cm²/sec at 20° was obtained for this virus. This figure is probably within ± 5 % of the true value, an assumption based on previous data obtained when the method was applied to the study of the diffusion constants of a variety of proteins.

INTRODUCTION

In a previous communication, VAN REGENMORTEL[1], reported on the determination of the diffusion constant of TYMV by the double gel diffusion technique of POLSON[2]. The value found by him agreed very well with the figure obtained by MARKHAM[3], who used the method of free diffusion in conjunction with the well known Lamm scale method.

Abbreviation: TYMV, turnip yellow Mosaic virus.

VAN REGENMORTEL conducted his experiments in 0.5 % agar gel in saline and as it appeared that this concentration of agar had no influence on the relative diffusion constant of virus and precipitating antibody, it was logical to assume that this concentration would also have no influence on the relative diffusion constants of poliovirus and its precipitating antibody. This assumption is based on the similarity in particle sizes of TYMV and poliovirus.

MATERIAL AND METHODS

The source of virus was a crude 1000 fold concentrate of infected monkey kidney tissue culture fluid of the Collans strain of Type 2 poliovirus which had been kept in the frozen state at —20° since 1955. Prior to storage the titre was $10^{9.8}$ $TCID_{150}$/0.5 ml. The virus was partially purified from 5 ml of this concentrate by three cycles of differential ultracentrifugation; 12,000 rev./min for 10 min to remove coarse tissue debris, 30,000 rev./min for 90 min to bring down the virus in a pellet and 8,000 rev./min for 10 min to remove coagulated matter from the redispersed pellet.

The immune serum used in the gel diffusion experiments was obtained from a monkey which had been hyperimmunized against the homologous antigen.

The diffusion experiments were conducted in a precision glass precipitin apparatus supplied by Messrs. L.K.B. Produkter (Stockholm), and in a similar apparatus made of perspex. Measurements on the positions and widths of the bands were made with a Hilger Watts microcomparator.

RESULTS

In Fig. 1a and b, are given photographs of the "perspex" gel diffusion apparatus after the diffusion process had proceeded for 8 and 60 days respectively. The white

Fig. 1a. Photograph of gel precipitin apparatus eight days after start of experiment. Antibody at constant concentration placed in cups in bottom of apparatus in liquid state, neutral agar layer in central tubes and falling twofold dilutions of antigen in upper cavities. Note minimum band width in region of fourth column.

Fig. 1b. Photograph of gel precipitin apparatus 60 days after start of experiment. Note the broadening of bands. Band in fourth column still narrowest. Additional bands, probably artefacts, produced after too long standing.

lines across the tubes in the central section in (a) are the precipitin bands which formed in 0.5 % agar through the reaction of serial two-fold dilutions of the virus suspension in the upper cavities, with a 1 in 10 dilution of the precipitating antibody in the cavities in the lower part of the apparatus. The linear relationship between antigen concentration and distance of the precipitin band from the antigen menisci can be clearly seen. In (b) is shown to what extent the bands have broadened after 60 days of diffusion, and that in the region where optimal proportions exists between the reactants, the widening of the band was less than on either side of this region.

In Fig. 2 is given a set of results obtained with the "perspex" apparatus. The oblique lines across the diagram are the band distances from the antigen menisci after 4, 5, 12, 17 and 34 days of diffusion. This was done by plotting the distances from the antigen menisci to the centres of the precipitin bands against the antigen dilutions. The V-shaped curves represent the widths of the corresponding precipitin bands as a function of the antigen dilution. It will be seen that the series of oblique lines intersect at a point 5.14 mm from the antigen meniscus. The position of this point corresponds very well with the average position where the limbs of the V-shaped curves intersect. On the left-hand side of the point of intersection of the oblique lines there is clearly a linear relationship between $\log_2$ antigen dilution and band position for all the lines. To the right of this point, however, the linear relationship does not hold for all the lines but shows a definite departure from linearity. This deviation tends to become progressively larger the longer the observation period was extended. The non-linear relationship may be explained by the fact that while the

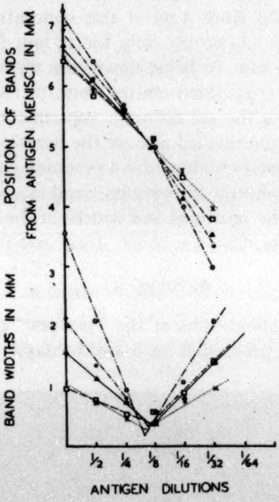

Fig. 2. Gel precipitin test on Type II poliovirus Collans strain. The dogainal curves show the average precipitin band positions, +, after 5; □, 6; △, 13; ●, 18, and ○, 35 days of diffusion plotted against the virus dilution. The V-shaped curves show corresponding variation of precipitin band widths plotted against virus dilution.

advancing edges of the precipitin bands remain sharp, the trailing edges tend to become diffuse as a result of redissolving of the precipitate when the antibody is in excess. When measuring the lines the centres are sought at positions further removed from the antigen menisci with the consequent deviation from linearity. Judging from other systems examined this source of error is not apparent when the antigen is in excess, or when both reactants are present in larger amounts.

Diffusion constant

At the position of intersection of the oblique lines, or where the minimum band width is located by the V-shaped curves, the two reactants are present in exact optimal proportions in their respective containers.

TABLE I

DETERMINATION OF X_g FROM THE V-SHAPED CURVES*

Time of diffusion in days	*Interpolated position on 5 days line*
5	5.2 mm
6	5.12 mm
13	5.10 mm
18	5.125 mm
35	5.175 mm
Average	5.14

* See Fig. 2.

In Table I are given the positions of minimum band widths as interpolated on the oblique straight line obtained during the initial stages of the experiment.

The following equation was used for calculating the diffusion constant, POLSON[2], OUCHTERLONY[4].

$$D_g = \left(\frac{X_g}{X_b}\right)^2 \cdot D_b \tag{1}$$

Where D_g and D_b are the diffusion constant of the virus and antibody, and X_g and X_b are the distances from the antigen and antibody menisci respectively where the precipitin band formed, when the reacting components are present in optimal proportions. $X_g + X_b = 14.94$ mm the length of the neutral agar column. D_b was assumed equal to $4.81 \cdot 10^{-7}$ cm^2/sec the value for horse tetanus antitoxin, LARGIER[5], and which was confirmed for the precipitin antibodies of Burnupena cincta haemocyanin prepared in the rat, guinea pig and rabbit[6]. Substituting $X_g = 5.14$, $X_b = 9.8$ and $D_b = 4.81 \cdot 10^{-7}$ cm^2/sec in eqn. (1) a diffusion constant of $1.32 \cdot 10^{-7}$ cm^2/sec at 20° was calculated.

TABLE II

DIFFUSION CONSTANT OF POLIOVIRUS DETERMINED BY THE DIOUBLE GEL DIFFUSION METHOD

Expt. No.	*D × 10⁷cm²/sec*
1	1.29
2	1.26
3	1.32
Average	1.29

In Table II are given the results obtained in three diffusion experiments on poliovirus. The average value, $1.29 \cdot 10^{-7}$ cm^2/sec is probably within ± 5 % of the true value as judged from previous results of diffusion measurements on proteins by this method[2].

Because of uncertainties regarding the density of the poliovirus particle (SCHAFFER AND SCHWERDT[7]), calculation of the molecular weight was not attempted at this stage.

DISCUSSION

The determination of the diffusion constants of viruses by the usual optical methods is dependent upon the availability of large amounts of pure virus. With few exceptions, notably the plant viruses, such quantities of infective material are usually not available. By applying the double gel diffusion technique of measuring diffusion constants a partial solution to the problem may be obtained. Although the degree of accuracy achieved by the latter method is not as high as that obtained by the optical methods, the relatively small amounts of infective material required for a diffusion experiment may be regarded as compensatory to the sacrifice of greater precision.

REFERENCES

[1] M. H. V. VAN REGENMORTEL, *Biochim. Biophys. Acta*, 34 (1959) 553.
[2] A. POLSON, *Biochim. Biophys. Acta*, 29 (1958) 426.
[3] R. MARKHAM, *Discussions Faraday Soc.*, 11 (1951) 221.
[4] O. OUCHTERLONY, *Arkiv. Kemi.*, B 26 (1949) 1.
[5] J. F. LARGIER, *Arch. Biochem. Biophys.*, 77 (1959) 350.
[6] A. POLSON, *Biochim. Biophys. Acta*, 43 (1960) 145.
[7] F. W. SCHAFFER AND C. E. SCHWERDT, *Advances in Virus Research*, 6 (1959) 159.

THE OXIDATION OF A REDUCED PYRIDINE NUCLEOTIDE ANALOG BY FLAVINS

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SUMMARY

The kinetics of the non-enzymic hydrogen transfer from 1-propyl-1,4-dihydronicotinamide to riboflavin have been studied. The riboflavin anion does not react, but the cation formed by protonation of the isoalloxazine nucleus is estimated to react 10^4 times faster than neutral riboflavin. Oxidation rates with several other flavins are compared.

Substituents on the ring nitrogen of the dihydronicotinamide which decrease the electron density of the ring cause a decrease in the oxidation rate.

Oxidation is more rapid the more polar the solvent, and the higher the ionic strength.

The rate of oxidation of the 4-deuterated 1-propyl-1,4-dihydronicotinamide is 3.2 times slower than that of the diprotiated form. Thus a hydrogen atom is removed in the rate limiting step of the oxidation.

The results are consistent with oxidation by a hydride ion transfer mechanism.

INTRODUCTION

Although riboflavin (I) in its coenzyme forms functions in many biological oxidation processes, little is known about the detailed mechanisms of these oxidation–reduction reactions. Riboflavin can be reduced to a stable semiquinone[1–3] and it is often assumed that the enzyme reactions involving flavin coenzymes are 1-electron transfer reactions. However, a hydride ion transfer mechanism may be just as plausible. The exact site at which hydrogen is transferred onto the flavin from the substrate is also uncertain. The possible importance of molecular complex formation prior to hydrogen transfer[4,5] needs clarification.

Of the many riboflavin dependent reactions, the oxidation of DPNH by riboflavin-containing enzymes of the electron transport chain of mitochondria is of special interest. The phosphorylation of adenosine diphosphate to adenosine triphosphate appears to be intimately coupled to this oxidation reaction. We have undertaken a study of the non-enzymic oxidation of DPNH by riboflavin in the hope of answering

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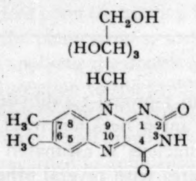
Abbreviations: DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide; NPrNH, 1-propyl-1,4-dihydronicotinamide.

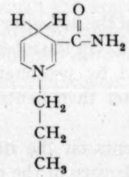
[*] From the Ph. D. thesis of Clarence H. Suelter, Iowa State University of Science and Technology, 1959.

some questions about the detailed reaction mechanism, and of perhaps gaining some insight relevant to the mechanism of oxidative phosphorylation.

The non-enzymic oxidation of DPNH and DPNH analogs by riboflavin occurs readily at room temperatures[6–8] SINGER AND KEARNEY[6] report that the reaction is first order in both DPNH and in riboflavin, and that the rate of reaction increases with decreasing pH.

Because of the complexity of the DPNH molecule, we have used an analog, NPrNH (II) in most of our experiments. This paper is a report of some of the details of the oxidation of NPrNH by riboflavin. Possible mechanisms for the reaction will be discussed.





I. Riboflavin(6,7-dimethyl-9-ribitylisoalloxazine) II. N-propyl-1,4-dihydronicotinamide(NPrNH)

METHODS AND MATERIALS

Kinetics

Reaction rates were measured through the use of a linear recorder (Varian Associates, Model G-10) attached to a Model DU Beckman Spectrophotometer which was equipped with a thermostatic cell compartment. In a typical experiment the buffer, riboflavin solution and other components which were previously equilibrated to the desired temperature were pipetted into a standard 3 ml rectangular silica cell and the volume was brought to 3 ml with H_2O. To initiate the reaction, 0.1 ml of the DPNH analog was added with an adder-mixer[9] and the O.D. was recorded at 360 mμ against a blank containing all reactants except NPrNH. Using this technique, measurements could be obtained within 8–10 sec after addition of the reduced analog. For each series of experiments (conducted on the same day) a fresh solution of the DPNH analog was prepared with water adjusted to pH 8–10; a fresh riboflavin solution was also prepared for each day's determinations. The pH of each reaction mixture was determined with a Beckman pH meter.

Deuterium analysis

The deuterium content of the [4-^{2}H]NPrNH was determined by burning the compound in dry oxygen, isolating the water produced, purifying[10] and analyzing for deuterium by the falling drop method[11]. The exact procedures and equipment are described by WARKENTIN[12].

Ionization constants

The apparent acidic dissociation constants of riboflavin and its analogs were determined spectrophotometrically. Measurements were conducted on approx. 10^{-4} M flavin solutions in 0.1 ionic strength buffers (except at high acid concentrations where the ionic strength exceeded 0.1). The following buffers were used: pH 0–2, HCl; pH 3–5, formate; pH 5–6.5, acetate; pH 7–8.5, triethanolamine; pH 8.5–10, ethanolamine or borate; pH 10.5–12, ethylamine or carbonate. For determination of K_1 (dissociation of riboflavin cation) absorbancies were measured at 400 and 450 mμ, and for determination of K_2 at 340 and 360 mμ. The experimental data were fitted with a theoretical titration curve, the midpoint of which determined the pK value. Since pK_1 is very low (except for 6,7-dimethyl-9-formylmethylisoalloxazine) and pH measurements could be made only to 0, the limiting absorbancies of the flavin cations needed for calculation of the theoretical titration curve had to be assumed. These assumed absorbancies were adjusted by trial and error until the theoretical curve was obtained which fit the data best.

MATERIALS

Nicotinamide-1-propochloride was prepared by the method which HOLMAN AND WIEGAND[13] describe for the 1-methochloride. 18.5 g of nicotinamide (Eastman Organic) in 30 ml of absolute methanol was refluxed with 27.5 g *n*-propyl iodide (Eastman Organic) for 10 h. The resulting quaternary iodide was converted to the chloride salt with a small excess of freshly prepared silver chloride. The compound was recrystallized from absolute ethanol. Yield 18.4 g (60 %); m.p. 192–194°; a_M $3.94 \cdot 10^3$ l/mole-cm at 265 mμ.

1-Propyl-1,4-dihydronicotinamide

Following a procedure of KARRER *et al.*[14], a solution of 4.3 g of nicotinamide-1-propochloride in 50 ml of H_2O was made alkaline by the addition of 15 g of anhydrous Na_2CO_3. The solution was placed in an ice bath and 14 g of sodium dithionite ($Na_2S_2O_4$) was added in small portions over a period of 10 min. The reaction was then stirred and SO_2 was removed by a stream of N_2 for 2 h. The reduced product was extracted with several portions of $CHCl_3$ totaling 500 ml. The $CHCl_3$ was removed *in vacuo* and the product was recrystallized as needed from reagent grade ether containing 0.5 % (by volume) water. The compound darkened rapidly when kept at room temperature, and hence, was stored in a desiccator at —25°. The compound melted at 91–91.5°, reported [15] 91–92°. a_M, $7.06 \cdot 10^3$ l/mole-cm at 360 mμ in H_2O, reported[16] $7.15 \cdot 10^3$ in ethanol.

[4-^{2}H]NPrNH

To 3 g of nicotinamide-1-propochloride in 80 ml of 99.5 % D_2O (Liquid Carbonic) at approx. 4° was added 960 mg of KCN. The solution was placed in a refrigerator (4°) for 2 h during which time it became quite dark in color. Hydrochloric acid (6 N) was then added dropwise with vigorous agitation until pH 7 was attained. During the latter operation and for an additional twenty minutes, N_2 was bubbled through the solution to remove all HCN. The preparation was evaporated to dryness *in vacuo* and was then taken up in 25 ml of 99.5 % D_2O for reduction. This solution was made basic by addition of 3.75 g Na_2CO_3. It was then reduced after placing it in an ice bath by the addition of 8 g of sodium dithionite in small portions over a 10-min period.

Nitrogen was bubbled through the solution for 2 h, the precipitated NPrNH was filtered off and additional NPrNH was extracted from the filtrate with several portions of $CHCl_3$ totaling 300 ml. The $CHCl_3$ was removed *in vacuo* and the [4-^{2}H]NPrNH was recrystallized from reagent grade ether containing 0.5 % H_2O by volume.

1-benzyl-3-acetyl-1,4-dihydropyridine was prepared by reduction of 1-benzyl-3-acetyl pyridinium chloride as described by ANDERSON AND BERKELHAMMER[16]. a_M, $1.04 \cdot 10^4$ at 380 mμ (λ_{max}) in 10 % ethanol, reported[16] a_M, $1.04 \cdot 10^4$ at 371 mμ (λ_{max}) in ethanol.

1-benzyl-1,4-dihydronicotinamide was prepared by reduction of nicotinamide-1-benzyl chloride[16]. m.p. 110–112°, reported[16] 110–114°; a_M, $7.40 \cdot 10^4$ at 360 mμ in 12 % ethanol, reported[16] a_M, $7.42 \cdot 10^4$ at 355 mμ in ethanol.

Riboflavin was purchased from the Merck Chemical Co. It was recrystallized twice from 2 *N* acetic acid and stored in a desiccator over $Mg(ClO_4)_2$ in the dark. a_M, $1.22 \cdot 10^4$ at 450 mμ and $1.06 \cdot 10^4$ at 375 mμ. Riboflavin-5′-phosphate, donated by the Sigma Chem. Co., was used without further purification. a_M, $1.12 \cdot 10^4$ at 450 mμ. Isoriboflavin (5,6-dimethyl-9-ribitylisoalloxazine) was used as obtained from the California Corporation for Biochemical Research.

Lumiflavin was prepared according to the procedure of HEMMERICH *et al.*[17]. The product was recrystallized from 2 *N* acetic acid. Calcd. for $C_{13}H_{12}N_4O_2$: C, 60.93; H, 4.72 %; Found: C, 60.67; H, 4.85 %.

3-Methyllumiflavin was synthesized by the same method used for the preparation of lumiflavin. A suspension of 3 g of N-methyl-3,4-dimethyl-6-(*p*-carboxyphenyl)-azoaniline[17] and 2.6 g of 3-methylbarbituric acid[18] was refluxed in 10 ml of glacial acetic acid and 30 ml of *n*-butanol for 5 h. 1-methyl-lumiflavin is not formed under these conditions. An equal volume of ether was added to the reaction mixture and the red orange precipitate was removed by filtration. 3-methyllumiflavin was recrystallized from anhydrous methyl alcohol. Yield 1 g; m.p. 284–285° dec.; a_M, $3.96 \cdot 10^4$ at 265 mμ, $9.90 \cdot 10^3$ at 370 mμ and $1.21 \cdot 10^4$ at 450 mμ. Anal. Calcd. for $C_{14}N_{14}O_2$: C, 62.21; H, 5.22 %; Found: C, 62.17; H, 5.30 %.

6,7-dimethyl-9-formylmethylisoalloxazine was synthesized by the procedure of FALL AND PETERING[19]. The compound which was recrystallized from absolute methanol and dried under vacuum at 78° for 5 h contained 1 molecule of methanol and decomposed at 270.5–271°. a_M, $2.66 \cdot 10^4$ at 270 mμ, $9.21 \cdot 10^3$ at 375 mμ and $1.11 \cdot 10^4$ at 450 mμ. Calcd. for $C_{14}H_{12}N_4O_3 \cdot CH_3OH$: C, 56.95; H, 5.10 %; Found: C, 57.04; H, 4.99 %.

RESULTS

Kinetics

In studying the related oxidation of reduced DPN by riboflavin, SINGER AND KEARNEY[6] noted that the dihydroriboflavin formed during the reaction was immediately reoxidized by dissolved oxygen. This observation simplifies the kinetic study of oxidation reactions of riboflavin in aqueous solution because the reactions need not be performed under difficultly attaine danaerobic conditions. However, since riboflavin oxidized NPrNH about 160 times more rapidly than DPNH at pH 6.65 in triethanolamine, it is necessary to reconsider this point. The following facts indicate that the reoxidation of reduced riboflavin by oxygen is not rate-limiting in the reaction with NPrNH.

1. The absorption of riboflavin at 450 mμ does not change during a reaction. A comparison of the spectra of dihydroriboflavin[3] and oxidized riboflavin indicates that the formation of a significant concentration of dihydroriboflavin could be detected at this wavelength.

2. The rate of the oxidation of NPrNH is the same whether done in a solution which is equilibrated with the atmosphere or in one which has been aerated with pure O_2. The concentration of O_2 in these solutions is estimated to be $2.6 \cdot 10^{-4}$ M and $1.23 \cdot 10^{-3}$ M respectively.

3. The 2nd order rate constant obtained in an anaerobic solution was identical to that obtained in an aerobic solution*.

Since dihydroriboflavin is immediately reoxidized by oxygen, the measured rate is that of the oxidation of NPrNH by riboflavin. The observed kinetics are first order with respect to NPrNH under all conditions tested and the first order rate constant k_1 varies in direct proportion to the riboflavin concentration (Fig. 1). Therefore, a second order rate constant, k_2 can be computed by equation 1.

$$-\frac{d(\mathrm{NPrNH})}{dt} = k_1(\mathrm{NPrNH}) = k_2(\mathrm{NPrNH})\,(\mathrm{riboflavin}) \qquad (1)$$

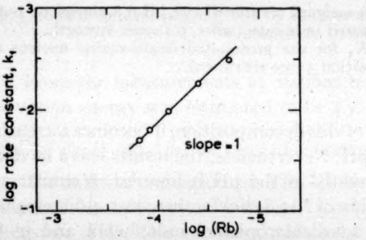


Fig. 1. Logarithm of the first order rate constant, k_1 (sec^{-1}) for the oxidation of NPrNH versus the logarithm of the riboflavin concentration, 25°.

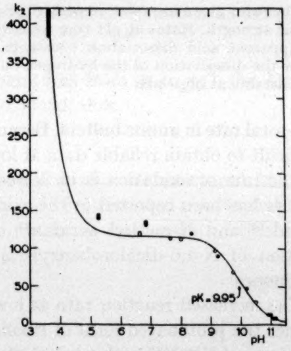


Fig. 2. The second order rate constant, k_2 (l·mole^{-1} sec^{-1} for oxidation of NPrNH by riboflavin versus pH, 0.1 ionic strength, 25°. ○, Nitrogen base buffers: pH 5–8.5, triethanolamine; pH 8.5–10.5, ethanolamine; pH 10.5–12, ethylamine. ●, Oxy-acid buffers: pH 3.6–6.5, acetic acid; pH 6.5–8.5, phosphoric acid; pH 8.5–10.5, boric acid; pH 10.5–12, bicarbonate.

The rate of oxidation versus *pH*

The second order rate constant k_2 is plotted against pH in Fig. 2. Above pH 7 the points fit the curve (solid line) which has been calculated upon the assumption that only the neutral form of riboflavin reacts at a measurable rate. This curve is governed by the dissociation of the hydrogen on position 3 of the flavin ring system with a pK of 9.95 (Others have reported values of 9.8 (see ref. 20) and 10.2 (see ref. 21).

Since the rate of the oxidation approaches zero at pH 12, the riboflavin anion either will not oxidize NPrNH or reacts at an extremely slow rate. Further confirmation of this conclusion is found in the fact that the rate of oxidation by 3-methyllumiflavin, which has no ionizable hydrogen, is just as large at pH 10.2 as at pH 6.8 (Table I).

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* We are indebted to Mr. D. CARR for the measurement of the rate constant in anaerobic solutions.

At lower pH values the loss of NPrNH through oxidation by riboflavin must be corrected for the acid-catalyzed decomposition of NPrNH. At pH 5 (10^{-4} *M* riboflavin) this correction amounts to 37 % of the total rate in acetate buffer and 7.2 % of the total rate in amine buffers. Because of this decomposition, it becomes increasingly difficult to obtain reliable data at low pH. Nevertheless, the results leave no doubt that the rate of oxidation is increased greatly as the pH is lowered. A similar acid catalysis has been reported in the oxidation of N-2,6-dichlorobenzyl-1,4-dihydronicotinamide[22] and N-methyl acridan[23] by 2,6-dichlorophenol indophenol and in the oxidation of N-2,6-dichlorobenzyl-1,4-dihydronicotinamide[20] and dihydrobenzene[24] by quinones.

TABLE I

COMPARISON OF RATES OF OXIDATION OF NPrNH AND p*K* VALUES FOR VARIOUS RIBOFLAVIN DERIVATIVES

Oxidant	*Rate* at pH 6.8*	*Rate at pH 10.2*	*pK_1***	*pK_2*
Riboflavin	1	0.35	0.12	9.95
Riboflavin-5′-phosphate	1.5	0.3	0.05	10.32
Isoriboflavin (5,6-dimethyl-9-ribityl-isoalloxazine)	2	0.8	— 1	10.0
Lumiflavin (6,7,9-trimethylisoalloxazine)	0.9	0.3		
3-methyllumiflavin	0.8	0.7	0.18	
6,7-dimethyl-9-formylmethylisoalloxazine	2	***	3.50	***
Flavin adenine dinucleotide	0.44			

* The rates given are relative with riboflavin assigned a value of 1 at pH 6.8 phosphate buffer, 0.1 ionic strength. Rates at pH 10.2 were measured in borate buffer, 0.1 ionic strength.

** Apparent acid dissociation constants, pK_1 for the protonated isoalloxazine nucleus and pK_2 for the dissociation of the hydrogen on position 3 (see METHODS).

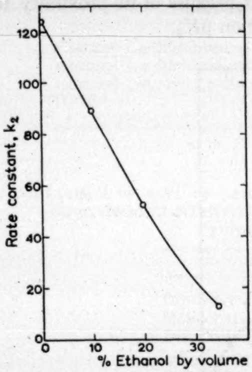
*** Unstable at high pH.

The increased reaction rate at low pH might result from a more rapid reaction of either the protonated form of riboflavin (pK = 0.12) with NPrNH, or of a protonated form of NPrNH with neutral riboflavin. The latter possibility is unlikely. When the decay curve of NPrNH at various pH values down to pH 3 is extrapolated to zero time, the same O.D. is observed, hence, the p*K* of the ring nitrogen of NPrNH is probably below pH 1.5. On this basis we estimate that the second order rate constant for the protonated riboflavin is $1 \cdot 10^6$, nearly 10^4 times greater than that for neutral riboflavin.

A third possible explanation for the increased reaction rate at low pH is that a general acid catalysis of the oxidation occurs. The increased rate of oxidation in phosphate buffer at pH 7 over that in amine buffers (Fig. 2) might result from general acid catalysis by $H_2PO_4^-$.

Effects of polarity and ionic strength and temperature of the reaction medium

The rate of oxidation is greatly reduced as the concentration of ethanol is increased (Fig. 3). The pH of the ethanolic solution were obtained with a Beckman pH meter which was standardized against 0.001 *M* HCl at the various ethanol concentrations used. This standard solution was assigned a pH of 3. The rate of oxidation increases with increasing ionic strength (Fig. 4), as would be expected for an ionic mechanism.



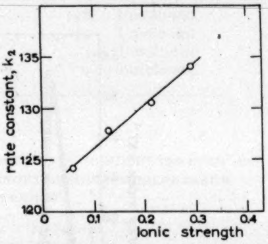


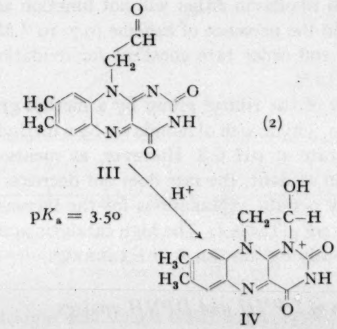
Fig. 4. Rate of oxidation of NPrNH by riboflavin in tris(hydroxymethyl)aminomethane buffer, pH 6.85, *versus* ionic strength.

Fig. 3. Rate of oxidation of NPrNH by riboflavin, *versus* ethanol concentration, 0.1 ionic strength phosphate buffer, pH 6.3, 20°.

From the measurements at various temperatures from 20 to 49°, the Arrhenius activation energy was estimated to be 4.7 ± 0.8 kcal/mole.

Effect of flavin structure on rates of oxidation

The rates of oxidation of NPrNH by several flavins are compared in Table I. Of particular interest is the high reactivity of 6,7-dimethyl-9-formylmethylisoalloxazine (III). This increased rate may be due to an interaction of the carbonyl group with the No. 1 nitrogen. Such interaction would be favored in the cation (IV), as indicated in eqn. 2. In support of this interaction is the high pK of 3.5 for the protonated form of 6,7-dimethyl-9-formylmethylisoalloxazine. The spectrum of the cation formed by portonation is essentially the same for both flavins (Fig. 5).



(2)

The cause for the increased rate of oxidation by riboflavin-5′-phosphate is uncertain, but may also be an acid catalysis by the protonated phosphate group. At pH 10 this hydrogen is not available and a slower rate is observed. Again the doubly charged ribityl phosphate anion may exert an effect because of its proximity to the isoalloxazine nucleus, both on the reaction rate and on pK_2.

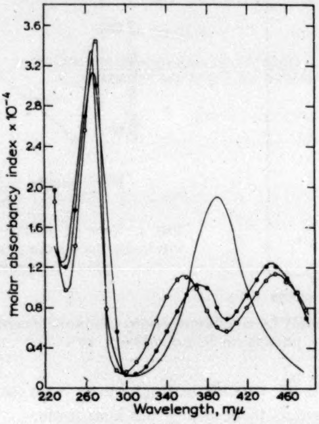


Fig. 5. Riboflavin spectra: O, anion; ●, neutral; —, calculated for riboflavin cation, from data at low pH and estimated pK value.

The slow rate of oxidation by flavin adenine dinucleotide is ascribed to the formation of an internal complex between riboflavin and adenine as described by Weber[25]. The complexed riboflavin either will not function as an oxidizing agent or reacts at a slower rate. In the presence of caffeine ($9.7 \cdot 10^{-3}$ M) a known complexing agent of riboflavin, the 2nd order rate constant for oxidation of NPrNH by riboflavin was decreased by 12 %.

Neither replacement of the ribityl group by a methyl group to give lumiflavin nor substitution of the No. 3 hydrogen of lumiflavin by a methyl group have significant effects on the reaction rate at pH 6.8. However, as mentioned on page 27, with 3-methyl lumiflavin as an oxidant, the rate does not decrease at pH 10.2.

We cannot offer any certain explanations for the increased rate observed with isoriboflavin and the low pK_1 (Table I). The high catalytic activity of this compound has been reported previously by Singer and Kearney[6].

Relative rates of oxidation of DPNH and DPNH analogs

From the data in Table II, which compares rates of oxidation for NPrNH, DPNH and two other analogs, we conclude that the stronger the electron withdrawing property of a group attached to the dihydropyridine ring, the slower the rate of oxidation. Wallenfels and Gellrich[22] have noted the same substituent effect during the oxidation of other DPNH analogs by 2,6-dichlorophenol indophenol.

TABLE II

COMPARISON OF RATE OF OXIDATION OF DPNH AND DPNH ANALOGS BY RIBOFLAVIN

Triethanolamine buffer, pH 6.65.

Compound	*k_2, 0.1 μ*
1 propyl-1,4-dihydronicotinamide	118 l/mole-sec
1 benzyl-1,4-dihydronicotinamide	29 l/mole-sec
1 benzyl-3-acetyl-1,4-dihydropyridine	3.7 l/mole-sec
DPNH	0.75 l/mole-sec

TABLE III

COMPARISON OF RATE OF OXIDATION OF DIPROTIATED, MONODEUTERATED AND DIDEUTERATED NPrNH, pH 7, TRIS(HYDROXYMETHYL)AMINOMETHANE BUFFER, 0.1 IONIC STRENGTH

Reactant	*k_2 l/mole-sec*
Diprotiated NPrNH	117
Monodeuterated NPrNH	106
Dideuterated NPrNH	37

Deuterium isotope effect

When the 4-deuterated-1-propyl-1,4-dihydro-nicotinamide which contained 1.41 deuterium atoms/mole was oxidized by riboflavin, the rate was not constant, but became slower as the oxidation proceeded indicating that the protiated derivative was being selectively oxidized. When the log of the 360 mμ absorbancy during a reaction was plotted versus time (Fig. 6), it was found that the last portion of the decay curve fell on a straight line which when extrapolated to zero time gave the theoretical starting concentration of dideuterated reactant. The second order rate constants for oxidation of the deuterated derivatives are compared in Table III.

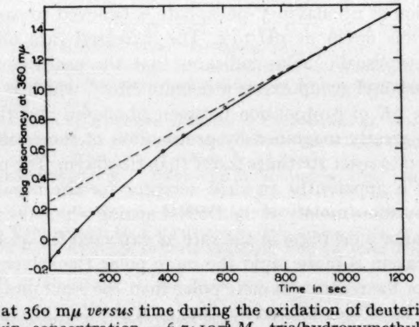


Fig. 6. Absorbancy at 360 mμ *versus* time during the oxidation of deuterium labelled NPrNH by riboflavin. Riboflavin concentration = $6.7 \cdot 10^{-5}$ *M*, tris(hydroxymethyl)aminomethane buffer, 0.1 ionic strength, pH 7, 25°.

The theoretical decay curve calculated with eqn. 3 agrees well with the experimental points (Fig. 6).

$$C_T = C_{HD}\,e^{-k_{HD}t} + C_{DD}\,e^{-k_{DD}t} \tag{3}$$

C_T = total concentration of monodeuterated and dideuterated NPrNH at time t, (C_T = Absorbancy at 360 mμ/$7.06 \cdot 10^3$), C_{HD} = initial concentration of monodeuterated NPrNH, C_{DD} = initial concentration of dideuterated NPrNH, and k_{HD}, k_{DD} = first order rate constants for oxidation of the monodeuterated and dideuterated product respectively. The deuterium isotope effect $k_{\mathrm{HH}}/k_{\mathrm{DD}}$ is 3.16 ± 0.05.

Effect of metal ions

Most metal ions tested (Cu^{++}, Cu^{+}, Zn^{++}, Mg^{++}, Mn^{++}, Fe^{++}, Co^{++}) either have no effect or exhibit a small inhibitory action on the reaction. However, Ag^{+} is a strong inhibitor, the rate in the presence of $1 \cdot 10^{-4}$ M Ag^{+} being decreased by a factor of 10. Silver is known to form a red complex of high stability with riboflavin[25].

Effect of light

Two recent reports[26, 27] indicate that the oxidation of reduced DPN by flavins is promoted by light. Careful measurements were made in this laboratory (by Mr. D. CARR) in which the reactants were kept in total darkness prior to and during the reaction, until the O.D. measurement was made. Rate constants for the oxidation of both NPrNH and DPNH by riboflavin were the same under these conditions as when the sample was continuously in the spectrophotometer beam, or intermittantly exposed to dim laboratory light. We believe, therefore that photochemical processes are not responsible for any of the results reported here.

DISCUSSION

The results presented can all be explained on the basis of an oxidation by hydride ion transfer. The riboflavin anion does not react since the negative charge on the molecule repels the further addition of a negative hydride ion. The doubly charged ribityl phosphate anion of riboflavin-5′-phosphate is believed to cause the decreased rate observed with this flavin at pH 10.2. The increased rate obtained with 6,7-dimethyl-9-formylmethylisoalloxazine indicates that the positive charge associated with the polarized carbonyl group exerts a definite effect, which is substantiated by the differences in the pK of protonation between riboflavin and the formyl-methyl analog. This effect is greatly magnified by protonation of the isoalloxazine nucleus. The cation is estimated to react 10^4 times faster than riboflavin. The positively charged isoalloxazine nucleus is apparently an ideal acceptor for the negative hydride ion. Substituents on the pyridine moiety of the DPNH analog which decrease the electron density of the ring cause a decrease in the rate as expected for an ionic mechanism.

The rate of oxidation is more rapid the more polar the solvent indicating that the transition state for the reaction is more polar than the reactants. Similar observations and conclusions have been offered by ABELES *et al.*[28] for the oxidation of DPNH analogs by thiobenzophenone, and by BRAUDE *et al.* who studied oxidation of dihydrobenzene by quinones[24].

An alternative mechanism has been advanced by SCHELLENBERG AND HELLERMAN[29] who have studied the oxidation of DPNH by free radical reagents of high oxidation potential. They conclude that two one-electron transfer steps are probably involved in the oxidation of DPNH by flavins. However, neither these experiments, nor the demonstration of a measurable concentration of semiquinone molecules in an active enzyme[30] rule out a hydride ion transfer mechanism. It is also clear that it would be difficult to distinguish between a hydride ion transfer and the slow transfer of a hydrogen atom occurring in a complex and followed by a rapid electron transfer.

Since the rate of oxidation of $[4\text{-}^{2}\text{H}]$NPrNH is 3.16 times slower than that of the diprotiated form, the hydrogen transfer step must be rate limiting.

No definite proof of molecular complex formation prior to hydrogen transfer was obtained during the course of this work. However, the low (4–5 kcal/mole) activation energy suggests that formation of such a complex with a negative enthalpy change may be involved. The formation of a weak complex with a negative enthalpy of formation between riboflavin phosphate and DPNH is suggested by spectrophotometric data of Isenberg and Szent-Györgyi[31] taken at —78°.

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REFERENCES

[1] R. Kuhn and T. Wagner-Jauregg, *Ber.*, 67 (1934) 361.
[2] L. Michaelis and G. Schwarzenbach, *J. Biol. Chem.*, 123 (1938) 527.
[3] H. Beinert, *J. Am. Chem. Soc.*, 78 (1956) 5323.
[4] B. Grabe, *Exptl. Cell Research*, 13 (1957) 588.
[5] B. Grabe, *Biochem. Biophys. Acta*, 30 (1958) 560.
[6] T. P. Singer and E. B. Kearney, *J. Biol. Chem.*, 183 (1950) 409.
[7] K. A. Schellenberg and L. Hellerman, *J. Biol. Chem.*, 231 (1958) 547.
[8] P. Karrer, B. H. Ringier, J. Buchi, H. Fritzche and U. Solmssen, *Helv. Chim. Acta*, 20 (1937) 55.
[9] P. D. Boyer and H. L. Segal, in W. D. McElroy and B. Glass, *A Symposium on the Mechanism of Enzyme Action*, Johns Hopkins Press, Baltimore, 1954, p. 520.
[10] A. S. Keston, D. Rittenberg and R. Schoenheimer, *J. Biol. Chem.*, 122 (1937) 687.
[11] I. Kirshenbaum, *Physical Properties and Analysis of Heavy Water*, McGraw Hill Book Co., Inc., New York, 1951, p. 324.
[12] J. Warkentin, *Addition of Hydrogen Bromide to Cyclohexadiene*, Unpublished Ph.D. Thesis, Library, Iowa State University of Science and Technology, Ames, 1959, p. 57-69.
[13] W. I. M. Holman and C. Wiegand, *Biochem. J.*, 43 (1948) 423.
[14] P. Karrer, G. Schwarzenbach, F. Benz and U. Solmssen, *Helv. Chim. Acta.*, 19 (1936) 811.
[15] D. Mauzerall and F. H. Westheimer, *J. Am. Chem. Soc.*, 77 (1955) 2261.
[16] A. G. Anderson, Jr. and G. Berkelhammer, *J. Am. Chem. Soc.*, 80 (1958) 992.
[17] P. Hemmerich, S. Fallab and H. Erlenmeyer, *Helv. Chim. Acta*, 39 (1956) 1242.
[18] J. A. VanAllen, in R. T. Arnold, *Organic Synthesis*, Vol. 32, John Wiley and Sons, Inc., New York, 1952, p. 45.
[19] H. H. Fall and H. G. Petering, *J. Am. Chem. Soc.*, 78 (1956) 377.
[20] L. Michaelis, M. P. Schubert and C. V. Smythe, *J. Biol. Chem.*, 116 (1936) 587.
[21] R. Kuhn and G. Moruzzi, *Ber.*, 67 (1934) 888.
[22] K. Wallenfels and M. Gellrich, *Ann. Chem. Liebigs.*, 621 (1959) 149.
[23] S. J. Leach, J. H. Baxendale and M. G. Evans, *Austral. J. Chem.*, 6 (1953) 409.
[24] E. A. Braude, L. M. Jackman and R. P. Linstead, *J. Chem. Soc.*, (1954) 3548, 3554; E. A Braude, A. G. Brook and R. P. Linstead, *J. Chem. Soc.*, (1954) 3574.
[25] G. Weber, *Biochem. J.*, 47 (1950) 114.
[26] W. R. Frisell and G. G. Mackenzie, *Proc. Natl. Acad. Sci., U.S.*, 45 (1959) 1568.
[27] L. P. Vernon, *Biochim. Biophys. Acta*, 36 (1959) 177.
[28] R. H. Abeles, R. F. Hutton and F. H. Westheimer, *J. Am. Chem. Soc.*, 79 (1957) 712.
[29] K. A. Schellenberg and L. Hellerman, *J. Biol. Chem.*, 231 (1958) 547.
[30] R. C. Bray, B. G. Malmstrom and T. Vanngard, *Biochem. J.*, 73 (1959) 193.
[31] I. Isenberg and A. Szent-Györgyi, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 1229.

INACTIVATION BY LIGHT OF THE PHOSPHORYLATIVE ACTIVITY OF CHLOROPLASTS*

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SUMMARY

The inactivation by light of the HILL reaction and the phosphorylating capacity of isolated chloroplasts has been studied. The results show that the ATP forming system is more sensitive than the electron transport system to light inactivation; conditions can be established under which a light pre-treatment decreases only 5 % the ability of chloroplasts to reduce TPN, while the ATP formation is decreased by 50 %. The light inactivation can be prevented to a large extent by the cofactors of photophosphorylation, vitamin K being the most efficient. ADP prevents the protection, and phosphate or arsenate restore it. The possible significance of these results is discussed.

INTRODUCTION

The finding that photosynthetic phosphorylation can be coupled to a HILL reaction[1] makes it possible to measure electron transport and ATP formation coupled to it independently of each other in isolated chloroplasts[2]. Such a possibility may be utilized in studying the mechanism of ATP formation in chloroplasts[2]. This report deals with a selectively greater inactivation by light of the ATP-forming system than of the HILL reaction.

METHODS

The chloroplasts were prepared from spinach leaves as described by JAGENDORF AND AVRON[3]. Chlorophyll was determined according to ARNON[4]. ATP formation was measured according to LINDBERG AND ERNSTER[5], as the incorporation of ^{32}P from P_i into ATP. PPNR was prepared as described by SAN PIETRO AND LANG[6]. TPN reduction was measured as the increase of O.D. at 340 mμ of a suspension of chloroplasts containing TPN and PPNR, against a blank to which no TPN was added. Ferricyanide reduction was measured by the decrease of O.D. at 420 mμ as described by AVRON *et al.*[7]. The chloroplast suspensions were illuminated in flasks agitated in an

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Abbreviations: ATP, ADP, adenosinetriphosphate, adenosinediphosphate; TPN, triphosphopyridinenucleotide; PPNR, photosynthetic pyridine nucleotide reductase; FMN, flavinmononucleotide; PMS, phenazinemethosulphate; P_i, inorganic orthophosphate.

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illuminated water bath, the light intensity being 4000 foot candles at the level of the flasks. The light was provided by incandescent lamps.

EXPERIMENTAL RESULTS

In the course of studies on photosynthetic phosphorylation, it was observed that ATP formation was linear only for a short period, no matter what cofactor was added to the reaction. These results are summarized in Fig. 1. It can be seen that with PMS, FMN or vitamin K_5 as catalysts, all added at saturating concentrations, the rate of ATP formation started to decrease after 10 min of illumination and was nearly suppressed after 30 min. The subsequent addition of any of the cofactors indicated above could not reactivate the system.

It was found further that pre-illumination of chloroplasts under nitrogen for periods of 15 min or more without the reagents needed for phosphorylation led to a

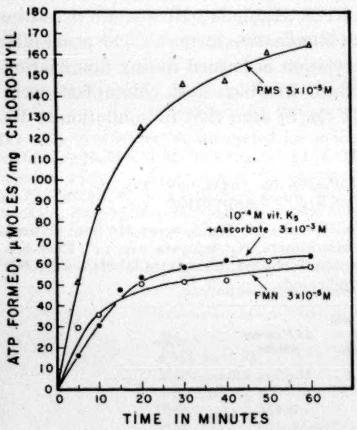

Fig. 1. Reaction mixture: NaCl 0.03 *M*; Tris 0.02 *M*, pH 8.0; $MgCl_2$ 0.003 *M*; ADP 0.005 *M*; Pi 0.005 *M*, labeled with ^{32}P. Final volume, 9 ml: N_2 atmosphere. Temperature 15°. Chloroplasts contained 0.108 mg of chlorophyll in the FMN and PMS experiment, and 0.126 mg in the vitamin K_5 experiment.

TABLE I

EFFECT OF PRE-ILLUMINATION OF CHLOROPLASTS ON THEIR CAPACITY TO COUPLE ATP FORMATION TO THE REDUCTION OF TPN

The chloroplast suspension was preincubated with KCl 0.03 *M*; Tris 0.02 *M*, pH 7.8; $MgCl_2$ 0.0033 *M*; ADP 0.0033 *M*, at 13°, under N_2 atmosphere. At the end of the time indicated, the following were added: TPN 1.6 μmoles, PPNR 20 units and 10 μmoles of phosphate, labeled with ^{32}P. Final volume 3 ml, pH 7.8. The chlorophyll content was 216 and 97 μg in Expts. 1 and 2. Reaction time, 10 min.

Experiment No.	*Pre-treatment conditions*	*TPNH formed μmoles*	*ATP formed μmoles*	*P/2e*
1	Dark 30 min	1.115	1.43	1.27
	Light 30 min	0.270	0.04	0.15
2	Dark 20 min	0.911	1.12	1.23
	Light 20 min	0.870	0.52	0.60

loss in their subsequent ability to carry out phosphorylation. The loss of activity due to pre-illumination was studied further in a system in which ATP formation is coupled to the reduction of TPN or of ferricyanide. Ih the former case, one has to deal with a "loosely coupled" phosphorylation, the rate of TPN reduction being unaffected by ADP, phosphate and Mg^{++}; in the latter, the phosphorylation is of the "tightly coupled" type, the rate of $[Fe(CN)_6]^{---}$ reduction being stimulated about 200 % by the addition of the phosphorylating reagents[7]. It can be seen in Table I that as a consequence of illumination of chloroplasts their capacity to form ATP is severely decreased while the reduction of TPN is little affected (Expt. 2). With a longer time of illumination the TPN reducing activity is also decreased about 75 %, but then the phosphorylation coupled to it is completely suppressed: the P/2e ratio is reduced from 1.27 to 0.15 (Expt. 1). It has to be observed that the light inactivation of the added PPNR, reported by GIOVANELLI AND SAN PIETRO[8], has no part in the phenomenon here described since PPNR was added after the pre-illumination.

In the HILL reaction with ferricyanide ATP formation is also inactivated by light at a faster rate than is ferricyanide reduction (Table II). Here again it can be seen that the P/2e ratio decreases as the time of illumination increases. The possibility that a stable, diffusible inhibitor of phosphorylation is formed during illumination of chloroplasts has been ruled out by experiments in which fresh chloroplasts were added to light inactivated ones (Table III). It can be seen that no inhibition is de-

TABLE II

EFFECT OF PRE-ILLUMINATION OF CHLOROPLASTS ON THEIR CAPACITY TO COUPLE ATP FORMATION TO $[Fe(CN)_6]^{---}$ REDUCTION

Preincubation: NaCl 0.03 *M*; Tris 0.02 *M*, pH 8.0; ADP 0.001 *M*; $MgCl_2$ 0.002 *M*; final volume 9 ml; chloroplasts containing 743 µg of chlorophyll. Atmosphere, N_2; temperature, 12°. Reaction mixture: same as preincubation, with 41 µg of chlorophyll in 3 ml; phosphate labeled with ^{32}P was added where indicated.

Pre-illumination time (min)	*$Fe(CN)_6^{---}$ reduced, in µmoles/2 min*		*ATP formed µmoles*	*P/2e*
	Without phosphate	*With phosphate 0.007 M*		
0	0.345	0.961	0.421	0.88
15	0.212	0.539	0.265	0.98
25	0.200	0.353	0.127	0.72
35	0.162	0.200	0.038	0.38

TABLE III

LACK OF FORMATION OF AN INHIBITOR OF PHOTOPHOSPHORYLATION DURING THE ILLUMINATION OF CHLOROPLASTS

Preincubation (in light or dark) as in Table I, with 15.4 µg of chlorophyll in 1.0 ml. After 30 min, 2.5 µmoles of $[^{32}P]$phosphate are added; FMN and non-preincubated chloroplasts where indicated. The reaction is carried on for 10 min, under N_2, at 15°.

Pre-illuminated chloroplasts	*Non-preincubated chloroplasts*	*ATP formed, µmoles/mg Chl. h.*	
		— FMN	*+ FMN $3 \cdot 10^{-5} M$*
+	—	0.4	3.1
+	+	11.0	133.5
—	+	10.5	138.5

tected, either in phosphorylation catalyzed by FMN or in the "endogenous" phosphorylation (that is, without any cofactor added). It is concluded from these experiments that the ATP-forming system of chloroplasts is inactivated by light at a faster rate than is their electron transport system.

Various reagents were added during the pre-illumination period in attempts to protect the chloroplasts from light inactivation (Table IV). No protection was afforded by ascorbate, cysteine or catalase and ethanol. Slight protection was afforded by high osmotic pressure. Of more interest is the protection afforded by PMS, pyocyanine, and vitamin K_5, all cofactors of cyclic photophosphorylation (Table IV). As can be seen, vitamin K_5 is the most effective of these cofactors in protecting the chloroplasts. Furthermore, its protection is just as efficient in red as in white light, while PMS protection is more efficient in red light than in white. Ferricyanide gives only a slight though reproducible protection.

The protection by the cofactors indicated in Table IV is suppressed or strongly reduced by the presence of ADP, while ATP has no effect (Table V). Separate experiments have shown that the presence of ADP alone during pre-incubation is harmful only when the chloroplasts are illuminated.

TABLE IV

PROTECTION OF CHLOROPLASTS FROM LIGHT INACTIVATION

The chloroplasts were preincubated for 30 min, at 14°, in N_2 atmosphere, in red light, in flasks containing NaCl 0.01 *M*; Tris 0.02 *M*, pH 8.0 and $MgCl_2$ 0.005 *M*. The suspension was then centrifuged at 18,000 × *g* for 10 min, the supernatant discarded, and the pellet washed with sucrose 0.4 *M*, containing Tris 0.02 *M*, pH 8.0, and NaCl 0.01 *M*, and finally resuspended in the same medium. Photo-phosphorylation was then measured in a system containing the same components as in Fig. 1 (PMS experiment), in a final volume of 1.5 ml. The reaction was allowed to proceed for 5 min.

Additions during preincubation	*ATP formed, µmoles/mg Chl. h.*
None	31
PMS 10^{-4} *M*	95
Pyocyanine 10^{-4} *M*	99
Vitamin K_5 10^{-4} *M*	149
Ascorbate 0.01 *M*	31
Catalase* + ethanol 0.01 *M*	32
None, preincubation in the dark	135

* The catalase added was enough to decompose 10 µmoles of H_2O_2/min.

TABLE V

EFFECT OF ADP ON THE PROTECTION FROM LIGHT INACTIVATION BY PMS

Preincubation, testing conditions and procedure as in Table IV, except for the absence of $MgCl_2$ during preincubation.

Additions during preincubation	*ATP formed, µmoles/mg Chl. h.*
None	4.3
PMS 10^{-4} *M*	68.4
PMS 10^{-4} *M* + ADP 0.003 *M* + $MgCl_2$ 0.005 *M*	15.5
PMS 10^{-4} *M* + ATP 0.005 *M* + $MgCl_2$ 0.005 *M*	73.6
None, preincubation in the dark	135.0

TABLE VI

EFFECTS OF ADP, ARSENATE AND PHOSPHATE ON THE PROTECTION FROM LIGHT INACTIVATION

Experiment No.	*Additions during preincubation**	*ATP formed moles/mg Chl. · hour*	*Increase over the control moles ATP/mg Chl. h.*
1	None	32.3	—
	Vitamin K_5	106.0	74
	Vitamin K_5 + ADP	75.0	43
	Vitamin K_5 + Pi	204.0	172
	Vitamin K_5 + ADP + Pi	117.0	85
	None, preincubation in the dark	157.5	125
2	None	59.0	—
	Vitamin K_5	212.0	153
	Vitamin K_5 + ADP	147.0	88
	Vitamin K_5 + Arsenate	254.0	195
	Vitamin K_5 + ADP + Arsenate	248.0	189
	None, preincubation in the dark	250.0	191

* Preincubation conditions: Vitamin K_5 10^{-4} *M*, and, where indicated, ADP 0.005 *M*; Pi 0.01 *M* and arsenate 0.001 *M*. Other conditions as in Table IV.

TABLE VII

EFFECT OF ADP CONCENTRATION ON K_5 PROTECTION FROM LIGHT INACTIVATION

*Additions during preincubation**		*ATP formed μmoles/mg Chl. · hour*	*Increase over the control μmoles ATP/mg Chl. h.*
Vitamin K_5	*ADP concentration mM*		
—	0.00	80.0	—
+	0.00	156.0	76
+	0.10	168.0	88
+	0.50	143.0	63
+	1.00	118.0	38
+	5.00	89.0	9

* Preincubation for 30 min, in white light. Vitamin K_5, where indicated, 10^{-4} *M*; ADP as indicated. Other conditions as in Table IV.

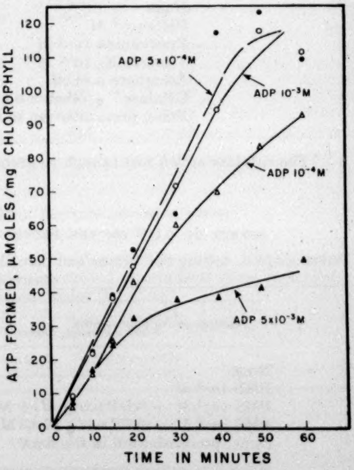

Fig. 2. Reaction mixture: NaCl 0.02 *M*; Tris 0.01 *M*; $MgCl_2$ 0.006 *M*; phosphate 0.005 *M* containing 300,000 counts/min of ^{32}P; hexokinase in large excess and glucose 0.02 *M*; vitamin K_5 10^{-4} *M*, ADP as indicated; pH 8.0. Final volume 8 ml; atmosphere: N_2·Chloroplasts containing 0.162 mg of chlorophyll.

The effect of ADP can be reversed by arsenate (Table VI). Phosphate seems also to be effective (Table VI), but the interpretation of its effect is complicated by the fact that phosphate alone, added during the light pre-incubation, provides considerable protection.

The concentration of ADP needed to reverse protection by cofactors shows a considerable variability with different chloroplasts preparations (compare Tables VI and VII); usually, however, the ADP effect is very strong or almost complete at $5 \cdot 10^{-3}$ *M*. The rate of phosphorylation, on the other hand, is saturated with about $5 \cdot 10^{-4}$ *M* ADP (Fig. 2). This suggested that the observed decline in rate of photophosphorylation with time (Fig. 1) might be due to a light inactivation facilitated by high concentrations of ADP, and that the use of lower concentrations of ADP might be able to prolong the linear part of the time course. Fig. 2 indicates that a linear time course can be achieved when the ADP concentration is not higher than $1 \cdot 10^{-3}$ *M*, and kept constant by means of the hexokinase–glucose system.

DISCUSSION

The data presented here demonstrate that light inactivates isolated chloroplasts and that the part of the system responsible for forming ATP is more sensitive than are the electron transport reactions *per se*. In fact, under the proper conditions one can observe a 50% decrease of the ATP formation coupled to TPN reduction, while TPN reduction itself is decreased by only 5% (Table I). An analogous phenomenon was observed with ferricyanide reduction.

The light inactivation here described is not due to the inactivation of PPNR reported by GIOVANELLI AND SAN PIETRO[8]. In fact, in our TPN reduction experiments PPNR was added after the pre-illumination, and therefore could not have been affected by the pre-treatment. Furthermore, the light inactivation of phosphorylation was observed also with ferricyanide reduction, and with cofactors such as PMS, FMN and vitamin K_1, while it is well known[9] that under such conditions photophosphorylation is independent of PPNR.

The mechanisms for light inactivation, for protection by redox cofactors (Table IV), for ADP reversal of the protection (Table V), and for phosphate or arsenate reversal of the ADP effect (Table VI) are all obscure. Some possibilities as to the nature of light inactivation can be ruled out, however. It has to be observed, first of all, that the phenomenon described here differs from the inactivation of PPNR by chloroplasts in the light[8] in two main respects: (a) PPNR inactivation is an aerobic effect, while our experiments were performed under nitrogen, and (b) PPNR photo-inactivation was prevented by ascorbate and cysteine, which had no effect here. It seems possible to rule out H_2O_2 as the damaging agent in the present case because of these two findings, and also because catalase failed to give any protection (Table IV). H_2O_2 formed in a "Mehler reaction"[10] was proposed by GIOVANELLI AND SAN PIETRO as responsible for PPNR inactivation[8].

Light inactivation might possibly be due to the photo-oxidant produced early in the HILL reaction; in this case the redox cofactors of cyclic electron transport might be able to reduce the photo-oxidant and so afford protection. This seems unlikely, however, because there is no reason why small amounts of the "photo-oxidant" could not be discharged by the evolution of oxygen. It seems reasonable to infer that the cofactors do afford protection by permitting electron flow to occur, whatever the mechanism of light-inactivation could be. For instance, light could be inactivating directly some chloroplast component, the inactivation occurring when the component is in one redox state but not the other. In that case, a steady electron flow caused by the cofactors could change the oxidation state of this component and "poise" it in the resistant form.

As to the effect of ADP and its reversal by arsenate or phosphate (Tables V and VI), one is tempted to suggest that the protection from light inactivation afforded by the redox cofactors cannot occur whenever ADP is bound to the "activating" system[2], whereas any conditions which will decompose the ADP–catalyst complex (as the presence of arsenate or phosphate according to the scheme proposed by AVRON AND JAGENDORF[2]) will restore the protecting effect. However, the ADP effect could also be due to the depletion of the internal pool of phosphate within the chloroplasts which occurs when the chloroplasts are incubated in the light with ADP and vitamin K_5 or PMS.

It seems unlikely that light-inactivation of the phosphorylation mechanism would occur *in vivo*, at least under the usual conditions of a sustained photosynthetic rate. Indeed, protection *in vitro* is afforded by cofactors for cyclic phosphorylation provided the ADP concentration is not abnormally high. Phosphate also protects from the ADP effect. These could be models for *in vivo* protection of plastids.

The possibility of inactivating selectively the ATP forming system of chloroplasts with little damage to the electron transport system could become a useful technique to study the phosphorylation mechanism itself.

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REFERENCES

[1] D. I. ARNON, F. R. WHATLEY AND M. B. ALLEN, *Science*, 127 (1958) 1026.
[2] M. AVRON AND A. T. JAGENDORF, *J. Biol. Chem.*, 234 (1959) 1315.
[3] A. T. JAGENDORF AND M. AVRON, *J. Biol. Chem.*, 231 (1958) 277.
[4] D. I. ARNON, *Plant Physiol.*, 24 (1949) 1.
[5] O. LINDBERG AND L. ERNSTER, in D. GLICK, *Methods in Biochemical Analysis*, Vol. III, Academic Press, 1956.
[6] A. SAN PIETRO AND H. LANG, *J. Biol. Chem.*, 231 (1958) 211.
[7] M. AVRON, D. W. KROGMANN AND A. T. JAGENDORF, *Biochim. Biophys. Acta*, 30 (1958) 144.
[8] J. GIOVANELLI AND A. SAN PIETRO, *Arch. Biochem. Biophys.*, 84 (1959) 471.
[9] A. T. JAGENDORF, *Brookhaven Symposia in Biol.*, 11 (1958) 236.
[10] A. H. MEHLER, *Arch. Biochem. Biophys.*, 33 (1951) 65.

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LIGHT INACTIVATION OF PHOTOPHOSPHORYLATION BY SWISS-CHARD CHLOROPLASTS

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SUMMARY

1. Photophosphorylation by swiss-chard chloroplasts was rapidly inactivated by preincubation of the reaction mixture in light. 50% inactivation was observed after 90 sec exposure to 160,000 lux.

2. The presence of phenazine methosulfate during the light preincubation period largely prevented the inactivation.

3. The inactivation was observed under a nitrogen atmosphere, when the preincubation was carried out under red light, with chloroplasts or chloroplast fragments, but not in the dark.

4. The quantitative requirements for light and phenazine-methosulfate during the preincubation and the following reaction periods were found similar to those of the photophosphorylative reaction proper.

5. No bleaching of chlorophyll could be observed during the light inactivation period. Addition of non-light-pretreated chloroplasts to pretreated ones did not restore their photophosphorylative abilities.

6. It is suggested that the light inactivation observed was the result of a destructive reaction of the normally produced early products in photophosphorylation.

INTRODUCTION

Light dependent phosphorylation was shown to be catalysed by swiss-chard chloroplasts at an extremely rapid rate[1]. Under optimal conditions rates of 2000 μmoles of ATP synthesized/mg chlorophyll/h could be demonstrated. It was noted, however, that the attainment of such rates was dependent, among others, on the presence of phenazine methosulfate as cofactor, the use of very high light intensities, and a very short reaction time (2 min or less). The unusually high activity of phenazine methosulfate, as compared with other cofactors, has been emphasized previously[2]. It is thought that this high activity might be related to the observations reported herewith.

The investigation of the light inactivation of photophosphorylation was prompted by the rapid decrease of photophosphorylative rate with time noted under the above mentioned optimal conditions[1]. Although it is still not possible to prevent

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Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; Tris, tris-(hydroxymethyl)aminomethane; PMS, phenazine methosulfate.

this decrease, it is hoped that the following might offer some clues for future investigations.

MATERIALS AND METHODS

For details of methods see ref. 1.

Preincubation experiments were run by exposing identical volumes of reaction mixtures to light for a specified time in the absence of one or more specified components (ADP was absent in all treatments); equal total volumes of materials were added in the dark as fast as possible, the flask contents were mixed, and returned to the light for a second specified time period. ATP production proceeded, therefore, only during the second exposure to light, since ADP was always absent during the initial exposure. It should be noted that by the second exposure to light all reaction mixtures had received the same identical components. The difference between treatments was limited, therefore, to changes as to which components were added before and which after the initial incubation in light.

Red light was produced by passing the white light through two layers of red cellophane paper. The paper had about 90% transmittance in the 650–700 mμ region, and less than 1% transmittance in the 400–570 mμ region. Unless otherwise indicated, white light was always used.

RESULTS

The decrease in the photophosphorylative activity of swiss-chard chloroplasts by pretreatment in light is shown in Fig. 1. It is evident that the high light intensity

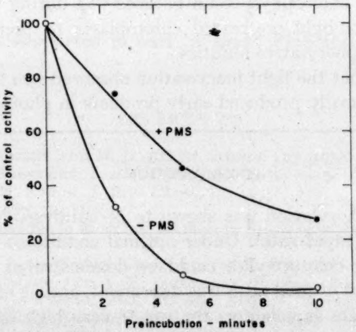

Fig. 1. Effect of preincubating chloroplasts in light for increasing periods in the presence or absence of PMS on their photophosphorylative abilities. Reaction mixture contained during the preincubation period the following components, in μmoles: Tris, pH 7.8, 45; NaCl, 60; $MgCl_2$, 12; sodium ascorbate, 30; phosphate, pH 7.8, 12 (containing 2–$5 \cdot 10^5$ counts/min of ^{32}P), PMS (only in + PMS samples), 0.09, and once-washed chloroplasts containing 18 μg chlorophyll, in a total volume of 2.80 ml. After preincubation in light of 160,000 lux for the specified times, 4 μmoles of ADP (and 0.09 μmoles of PMS in the samples which did not contain PMS during preincubation) were added in a total volume of 0.20 ml. The contents were mixed and exposed to 160,000 lux of light for 2 min. Trichloroacetic acid was added to a final concentration of 2%, the reaction mixtures centrifuged, and the supernatant analysed for its [^{32}P]ATP content as already described[1]. Temperature, 17°, Gas phase:air. 100 on the ordinate corresponds to an activity of 1200 μmoles ATP formed/mg chlorophyll/h.

caused rapid inactivation. 50 % inactivation, as compared to non-pretreated controls, was attained after only 90 sec of light pretreatment. Virtually total inactivation required only about 8 min of preincubation in the light.

Early in the studies of the phenomenon it became clear that PMS, the cofactor of the reaction, could largely prevent the inactivation process. Fig. 1 illustrates the inactivation observed in the presence and absence of PMS during the preincubation period. In the presence of PMS, 50 % inactivation was attained only after about 5 min of light pretreatment. The extent of inactivation, and of its prevention induced by the presence of PMS during preincubation, has been variable, but both effects have always been large and clear cut. In some preparations, the presence of PMS during the preincubation period brought about virtually complete protection from light inactivation. The data of Fig. 1 show a typical, rather than an extreme case.

Several other materials were tested as to their ability to show the same protective effect as PMS. The other cofactors tried, flavin mononucleotide, and menadione, showed small to no protective action. Ascorbic acid had some protective effect, but never as large as the one exhibited by PMS.

Expts. 1 and 3 in Table I illustrate that the results were totally dependent on light, in the presence of PMS. Some inactivation occurred, however, if PMS was absent during a dark preincubation. The latter was always small in relation to the inactivation caused by preincubation in the light (Expt. 3 in Table I). Although

TABLE I

EFFECT OF LIGHT AND GAS PHASE ON PHOTOINACTIVATION

Conditions as described under Fig. 1, except for variation, as indicated: Expt. 1, once washed chloroplasts containing 31 μg chlorophyll per flask. Control value: 530 μmoles ATP formed/mg chlorophyll/h. Expt. 2, darkness was obtained by completely covering reaction flasks with aluminum foil. Once washed chloroplasts containing 20 μg chlorophyll per flask. Control value: 600 μmoles ATP formed/mg chlorophyll/h. Gas phase was changed to nitrogen by bubbling prepurified nitrogen through a gas disperser into alkaline pyrogallol, then through water and then through the flasks. The nitrogen was passed for 5 min before the light was turned on, and continuously throughout the reaction period. Expt. 3, once washed chloroplasts containing 17 μg chlorophyll per flask. Control value, 1015 μmoles ATP formed/mg chlorophyll/h. Darkness attained as in Expt. 1. Expt. 4, once washed chloroplasts containing 35 μg chlorophyll per flask. Control value: 700 μmoles ATP formed/mg chlorophyll/h. Red light was produced as described under METHODS.

Expt. No.	*Conditions*						*% of control*
	During preincubation			*During reaction*		*Throughout*	
	Time (min)	*Illumination (lux)*	*Absent*	*Time (min)*	*Illumination (lux)*	*gas phase*	
1	20	Dark	ADP	5	45,000	Air	97
1	20	Dark	ADP + PMS	5	45,000	Air	87
2	20	45,000	ADP	5	45,000	Air	38
2	20	45,000	ADP + PMS	5	45,000	Air	5
2	20	45,000	ADP	5	45,000	Nitrogen	53
2	20	45,000	ADP + PMS	5	45,000	Nitrogen	41
3	5	Dark	ADP	5	160,000	Air	100
3	5	Dark	ADP + PMS	5	160,000	Air	72
3	5	160,000	ADP	5	160,000	Air	45
3	5	160,000	ADP + PMS	5	160,000	Air	15
4	5	160,000	ADP	5	160,000	Air	57
4	5	160,000	ADP + PMS	5	160,000	Air	18
4	5	Red, 160,000	ADP	5	Red, 160,000	Air	70
4	5	Red, 160,000	ADP + PMS	5	Red, 160,000	Air	44.

slightly depressed, the same general phenomena appeared when the reaction was run anaerobically, under a nitrogen atmosphere (Expt. 2 in Table I), or in red light in place of white light (Expt. 4 in Table I). It cannot be attributed, therefore, solely to photooxidation by free oxygen, or to action of light on pigments which do not absorb in the red region of the spectrum.

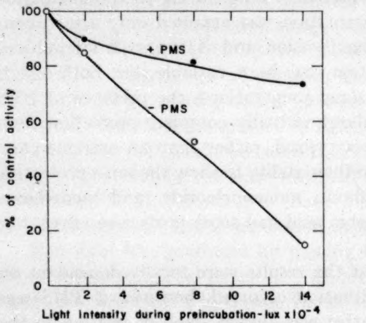

Fig. 2. Effect of light intensity during the preincubation period. Conditions as described under Fig. 1, except for the following: the light intensity during the preincubation period was varied as indicated. The light remained at 160,000 lux in all cases during the second exposure to light. Red light used throughout (see METHODS). Preincubation for 10 min. Reaction (second exposure) for 5 min. Once washed chloroplasts containing 36 μg chlorophyll per flask. 100 on the ordinate corresponds to an activity of 1050 μmoles ATP formed/mg chlorophyll/h.

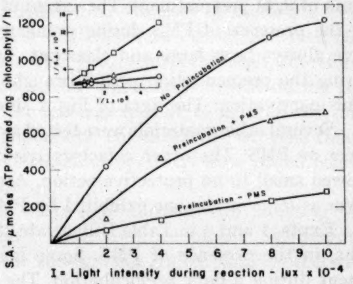

Fig. 3. Effect of light intensity during the reaction, after a preincubation period. Conditions as described under Fig. 1, except for the following: the light intensity was varied during the reaction (second exposure) as indicated. The light remained at 160,000 lux during the preincubation period. Preincubation for 5 min. Reaction for 5 min. Once-washed chloroplasts containing 32 μg chlorophyll per flask.

The same results were obtained when chloroplast fragments were used in place of whole chloroplasts, and when the chloroplasts were isolated from spinach, in place of swiss-chard.

The light-intensity requirements during the preincubation period are shown in Fig. 2. It can be seen that high light intensities were required for maximal inactivation. As shown previously[1], similar light intensities were necessary for maximal photophosphorylation in these chloroplasts. The high light intensity required was, however, in contrast with the light requirements for photoinactivation of the enzyme photosynthetic pyridine–nucleotide reductase. In the latter case saturation was reached at low light intensities[3].

The requirement for light after the preincubation period is recorded in Fig. 3. It is evident that the rates of the reaction were markedly decreased by the light pretreatment, but the amount of light necessary for maximal activity during the reaction has not changed markedly. This is interpreted to indicate that the main factor damaged during the light-preincubation period is closely associated with the initial light absorption reaction and not with later steps. Plotting the same data in the manner suggested by LUMRY *et al.*[4], as shown in the inset of Fig. 3, reemphasizes this conclusion. A change in slope in such a plot indicates interference with the light reaction, while a change in intercept indicates interference with a dark reaction.

If, as indicated above, the damage is caused mostly within the initial reactions, associated with light absorption, the effect should be relatively independent of temperature. Table II describes an experiment in which the preincubation was carried out at two different temperatures while the reaction was at the same temperature. When the chloroplasts were pretreated at 2°, in the absence of ADP and PMS, the inhibitory action was decreased as compared with pretreatment at 17°, but was nevertheless unquestionably present. When PMS was present during the preincubation period the damage was largely prevented at 17°, but not at all at 2°. It seems, therefore, that light inactivation was indeed mostly a photoreaction not involving chemical or enzymic reactions. The prevention of inactivation by PMS, on the other hand, must have involved such chemical or enzymic reactions.

TABLE II

EFFECT OF TEMPERATURE DURING THE PREILLUMINATION PERIOD

Conditions as described under Fig. 1, except for the following variations: All four flasks were exposed to 2° for 10 min, but only those marked 2° were illuminated at 160,000 lux (the other two being completely covered with aluminum foil). The temperature of the bath was now raised to 17° in the dark, the flasks marked 2° covered completely with aluminum foil, and the flasks marked 17° exposed to 160,000 lux for 10 min. ADP, or ADP + PMS, as indicated, was now added, the contents mixed, and all flasks exposed to 160,000 lux for a reaction period of 5 min. Once washed chloroplasts containing 32 μg chlorophyll per flask. Control value: 1500 μmoles ATP formed/mg chlorophyll/h.

Temperature during preillumination	*Absent during preincubation*	*% of control*
17°	ADP	75
17°	ADP + PMS	9
2°	ADP	30
2°	ADP + PMS	41

The effect of several concentrations of PMS in the prevention of photoinactivation is compared in Fig. 4 with the effect of the same concentrations on the photophosphorylative reaction proper. It can be seen that increasing concentrations of PMS reacted in a very similar fashion in the two functions, especially in the low concentration range. This would seem to indicate that PMS is reacting in both cases with the same intermediate within the chloroplasts, and that the two actions observed are essentially two different manifestations of the same basic interaction.

The effect of high intensity light in bleaching chlorophyll is well known[5]. However, the chlorophyll content of a reaction mixture, before and after a light incubation period which caused 76 % inactivation of photophosphorylation, showed no decrease whatsoever. Chlorophyll content was measured by the method of ARNON[6]. The light effect which was responsible for the inactivation of photophosphorylation was therefore not identical with that causing the bleaching reaction. It could, of course, be an initial phase in the bleaching process.

The possibility that light inactivated a component which could be added back, has also been tested. The results presented in Table III, indicate that the addition of non-pretreated chloroplasts, to pretreated (*i.e.* photoinactivated) ones, did not increase the photophosphorylative ability of the light pretreated chloroplasts. The amount of ATP formed in the reaction in which both types of chloroplasts were present (3rd line) was approximately equal to the sum of that formed by each type of chloroplasts by itself (1st and 2nd line). It did not approach the amount of ATP formed by the same amount of non-preincubated chloroplasts (4th line). The component which was inactivated could not, therefore, be restored by the addition of non-preincubated chloroplasts.

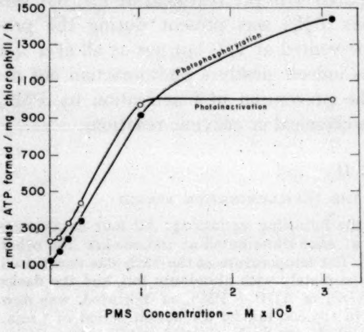

Fig. 4. Effect of PMS concentration on photoinactivation and photophosphorylation. In the photoinactivation experiments conditions were as described under Fig. 1, except for the PMS concentration during the preincubation period, which was varied as described. After the preincubation, additional PMS was added so that all flasks contained the identical optimal amount of PMS (0.09 μmoles per flask) during the following reaction period. Preincubation for 5 min. Reaction for 2 min. In the photophosphorylation experiments the [^{32}P]ATP formed after 2 min of reaction with the concentration of PMS indicated was measured. No preincubation was done in this case. Light, 160,000 lux. Once washed chloroplasts containing 25 μg chlorophyll per flask.

In other experiments, the addition of an extract from swiss-chard leaves which had photosynthetic pyridine-nucleotide reductase[3] activity, was also ineffective in reversing the light inactivation.

TABLE III

LACK OF REVERSAL OF PHOTOINACTIVATION BY ADDITIONAL CHLOROPLASTS

Conditions as described under Fig. 1, except for the indicated variations. Once washed chloroplasts containing 27 μg chlorophyll/0.1 ml in the 5-min preincubation experiment. Chloroplast fragments containing 22 μg chlorophyll/0.1 ml in the 10 min preincubation experiment. Preincubation at 160,000 lux for the times indicated. Reaction at the same light intensity for 5 min.

Added before preincubation	*Added after preincubation*	*μmoles ATP formed*	
		5 min preincubation	*10 min preincubation*
0.1 ml chloroplasts	ADP + PMS	0.77	0.37
none	ADP + PMS + 0.1 ml chloroplasts	2.61	2.07
0.1 ml chloroplasts	ADP + PMS + 0.1 ml chloroplasts	3.54	2.74
none	ADP + PMS + 0.2 ml chloroplasts	4.22	4.22

DISCUSSION

The observations reported herewith emphasize the double role played by light in photophosphorylation. It is, of course, a necessary component of the reaction, but at the same time it causes a rapid and as yet irreversible inactivation of the chloroplasts. The two effects can be measured independent of each other, as was done throughout this presentation. It could be shown, however, that the same inactivation occurred during the progress of photophosphorylation. This can be clearly seen in

Fig. 5, where four simultaneous reactions were run, each receiving its ADP at a different time. When the rate of the reactions which received their ADP at later than zero-time was replotted on the control curve, it was evident that they conformed well with its shape. It is thus apparent that the inactivation was not dependent upon whether or not phosphorylation proceeded simultaneously with it.

The light inactivation reaction explains the necessity of running the reaction for short periods of time in order to realize the very high rates of photophosphorylation previously reported[1]. At the high intensities of light necessary to attain those rates, inactivation by light must have been extremely rapid, causing a sharp decline in the rate as time progressed.

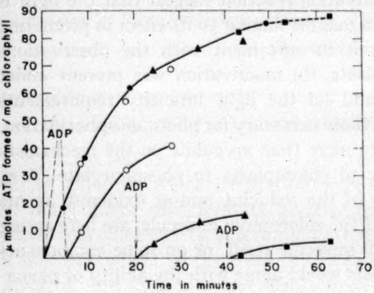

Fig. 5. Comparison of photoinactivation during photophosphorylation and after light-preincubation. Conditions as described under Fig. 1, except for ADP addition at the times marked. Light, 100,000 lux. Chloroplast fragments containing 15 μg chlorophyll per flask.

The light inactivation of photophosphorylation studied may well be the initial reaction damaged during the "induction phase" in the experiments reported by KANDLER AND SIRONVAL[5,7]. The earliest effect which they could detect was a decrease of the photosynthetic rate after 10 min illumination of *chlorella* cells, at 100,000 lux. They also observed a large decrease in the phosphorylative rate of whole chlorella cells after preincubation in the light for 2.5 h (shorter preincubation times were not reported). The latter decrease was not related to a decrease in the oxygen absorption capacity of the cells and it was therefore suggested that "oxidative phosphorylation is very likely a main site of action of photooxidation during the induction phase". In view of the results presented here, it would seem more likely to regard photophosphorylation, rather than oxidative phosphorylation, as the phosphorylative step damaged early in the photoinactivation process. The low rate of phosphorylation which remained in their experiments, after 2.5 h of illumination at 100,000 lux, could indeed be due to oxidative-phosphorylation which was not damaged by the preillumination period.

The ability of PMS to partially prevent the inactivation might well be related to its outstanding ability as a cofactor in photophosphorylation. The similarity in its concentration requirements in both processes (Fig. 4) indicates that it might interact with the same components in the chloroplasts in performing both functions. The ability of PMS to prevent the slow inactivation of the chloroplasts in the dark (Table I), is also of interest in view of the difficulties encountered in maintaining chloroplasts of high phosphorylative capacity for long periods of time.

In this connection, it is of interest to compare the action of PMS with that of carotenoids in photosynthetic bacteria. FULLER AND ANDERSON[8] observed a large photoinactivation of the photophosphorylative reaction in chromatophores prepared from carotenoidless bacteria. The normal bacteria were not nearly as sensitive. It would seem, therefore, that the carotenoids serve a function similar to that exhibited by PMS in this communication. Although added carotene did not have any protective effect similar to PMS in our chloroplast system, it could, of course, have such a protective function *in vivo*. In that case, the light inactivation studied here will essentially be similar in mechanism to that studied in photosynthetic bacteria[8,9].

The characteristics of the photo-inactivation reaction suggest that the light is acting through the chlorophyll molecule in a manner similar to its effect in promoting photophosphorylation. Such a conclusion is in agreement with the observations that (a) red light inactivated the chloroplasts, (b) inactivation was present under nitrogen, as was photophosphorylation, and (c) the light intensity requirements for the inactivation reaction were similar to those necessary for photophosphorylation.

The data at hand do not permit one to more than speculate on the mechanism through which light depresses the ability of chloroplasts to phosphorylate. It is, however, reasonable to assume that some of the reducing and/or oxidizing agents generated very early in the photoreaction of the chlorophyll molecule, are here acting in a destructive manner on the chlorophyll molecule itself, or on some components closely associated with it. Such an hypothesis would agree with the ability of phenazine methosulfate to prevent the inactivation, since it is most likely capable of reacting with these reducing or oxidizing agents.

Finally, it should be pointed out, that light inactivated chloroplasts could serve as a very convenient source of the enzymic complex of chloroplasts devoid of its ability to catalyse light dependent reactions. Such preparations may prove useful in the further study of the properties of the non-light-dependent enzymic processes of chloroplasts.

ACKNOWLEDGEMENT

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REFERENCES

[1] M. Avron, *Biochim. Biophys. Acta*, 40 (1960) 257.
[2] A. T. Jagendorf and M. Avron, *J. Biol. Chem.*, 231 (1958) 277.
[3] J. Giovanelli and A. San-Pietro, *Arch. Biochem. Biophys.*, 84 (1959) 471.
[4] R. Lumry, J. D. Spikes and H. Eyring, *Ann. Rev. Plant. Physiol.*, 5 (1954) 298.
[5] C. Sironval and O. Kandler, *Biochim. Biophys. Acta*, 29 (1958) 359.
[6] D. I. Arnon, *Plant Physiol.*, 24 (1949) 1.
[7] O. Kandler and C. Sironval, *Biochim. Biophys. Acta*, 33 (1959) 207.
[8] R. C. Fuller and I. C. Anderson, *Nature*, 181 (1958) 253.
[9] G. Cohen-Bazire and R. Y. Stanier, *Nature*, 181 (1958) 250.

CONCENTRATIONS OF PHOSPHATIDES AND GLYCOLIPIDS IN LEAVES AND CHLOROPLASTS

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SUMMARY

Deacylated leaf and chloroplast lipids were separated by twodimensional chromatography. Quantitative determinations were made of the 4 major phosphate diesters, and of the 3 major carbohydrate–glycerol compounds. From the comparison of whole leaves and chloroplasts, and similarly from the comparison of green and yellow leaves, it was concluded that the green chloroplasts are particularly enriched in phosphatidylglycerol, and in mono- and digalactosyl lipids, the latter compounds occurring in concentrations approaching or exceeding 10^{-2} *M*. Lecithin, cephalin, phosphatidylinositol, and the plant sulfolipid were not specifically linked to the photosynthetic apparatus.

INTRODUCTION

After the recognition of phospholipids and glycolipids as important constituents of the photosynthetic apparatus[1–3], it became of interest to further establish the quantities involved by applying techniques other than those using radioactive tracers or neutron activation. This was achieved by a slight modification of the technique used by BENSON *et al.*[3], and the results confirm the particularly close association of phosphatidyl glycerol and of the galactolipids with the photosynthetic apparatus.

MATERIALS AND METHODS

Leaves and chloroplasts from spinach, *Spinacia oleracea* and sugarbeet, *Beta vulgaris* were used. Spinach chloroplasts were isolated in 0.4 *M* sucrose, 0.01 *M* NaCl, 0.05 *M* Tris pH 7.8 (see ref. 4); beet chloroplasts were isolated in 0.35 *M* NaCl, 0.001 *M* $MgSO_4$, 0.067 *M* Tris pH 7.4 (see ref. 5).

Green and yellow leaves of two other species were compared: elder, *Sambucus nigra*, and bean, *Phaseolus vulgaris*. From the first species normal green plants were available and an *aurea* form, in which the chlorophyll is abnormally sensitive to light.

Abbreviations: GP, glycerophosphate; GPC, glycerophosphoryl choline; GPE, glycerophosphoryl ethanolamine; GPG, glycerophosphoryl glycerol; GPI, glycerophosphoryl inositol; GPS, glycerophosphoryl serine; GPGPG, 1,3-diglycerophosphoryl glycerol; G-gal, monogalactosyl glycerol; G-gal-gal, digalactosyl glycerol; G-gal-SO_3, glyceryl galactoside sulfonic acid.

* 201st communication; 74th communication on photosynthesis.

In this variety especially the outer leaves, which are exposed to full sunlight are very poor in chlorophyll, survival probably depending on photosynthesis by the less exposed shade leaves. Microscopic examination shows that the yellow leaves have reduced palissade parenchyma, and few chloroplasts. It can be assumed that the photosynthetic apparatus in these leaves is damaged by excess of light.

In the experiments with bean, seedlings were used which had been grown in red light at 3 different daylengths, viz. 0.5, 2 and 8 h daily. The shortest illumination induced very little chlorophyll formation, but allowed the plants to reach normal shape; especially stem length and leaf size were normal, in contrast to fully dark grown plants. The plants grown at the short daylengths can be assumed to be poor in chlorophyll owing to lack of light.

Leaves separated from petioles, or packed chloroplasts were killed in boiling 95 % ethanol and further extracted with boiling ethanol–toluene mixtures, until colourless. An aliquot from the combined supernates was diluted with ethanol for the estimation of chlorophyll, using the extinction coefficients given by HEIERLE[6]. The bulk of the extract was concentrated *in vacuo*, lipids were taken up in chloroform, and thoroughly washed with water. The lipid phase was dried, and taken up in methanol–toluene. An aliquot was used for the determination of total lipid P, the remainder was hydrolysed by the addition of an equal volume of methanol–0.2 *N* KOH, and incubation at 37° for 15 min. Water and chloroform were added to the hydrolyzate, and the supernatant, containing the water soluble deacylated lipids was neutralized with Amberlite IR-120, H-form. Since pigments and fatty acids still are present in the supernate at this stage, the latter is again thoroughly washed with chloroform. Emulsions can be broken by centrifugation, or by the addition of ethanol. In the latter case, practically colourless solutions can be obtained, but some P stays in the chloroform phase. This removal of fatty material was desirable, since, in its presence, a very low limit is set upon the amounts of substance that can be separated by paper chromatography.

The water phase is dried *in vacuo*, taken up in 50 % ethanol, and applied to the origin of paper chromatograms. Whatman No. 4 paper was used throughout, and

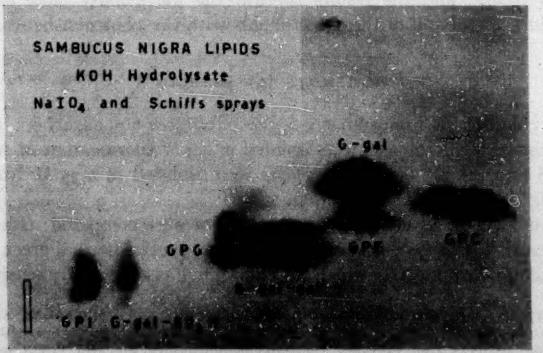

Fig. 1. Paper chromatogram of deacylated lipids of *Sambucus nigra cv. aurea*. Development with phenol–water, left to right, and with butanol–propionic acid–water upwards.

solvents used were phenol–water (3:1), and butanol–propionic acid–water (14:7:10, v/v). Up to 150 μg P, equivalent to about 1.5 g of leaf, or about 0.7 ml of packed chloroplasts can be conveniently separated in this way.

Phosphates were detected with molybdate reagent[7], followed by 10 min heating at 70° and u.v. irradiation. Traces of phenol are particularly interfering, and 16–24 h drying after the phenol run is therefore recommended.

The deacylation products of the galactolipids, as well as those of the phosphatides can also be detected by periodate-Schiff reagent[8]. A typical chromatogram obtained in this way, is shown in Fig. 1. For the identification of the P containing spots, see ref. 1; for the identification of the carbohydrate containing spots, see ref. 3. For quantitative P determinations, the spots or corresponding areas of duplicate chromatograms were eluted. Total P was determined after destruction of the eluate with sulfuric and nitric acids. Recovery usually was from 60–90 % of the amount applied to the origin. The galactose containing spots were always eluted with water from the duplicate chromatogram. 1-ml aliquots were taken for total carbohydrate determination using the anthrone method[9].

EXPERIMENTAL RESULTS AND DISCUSSION

The averaged results of these observations are given in Table I. From the ratio of chlorophyll concentrations in leaf and chloroplasts an estimate of the volume percent of chloroplasts in the leaves[§] can be obtained. Hence, the amounts of chloroplast phosphatides and glycolipids per unit volume of leaves can be calculated, and com-

TABLE I

PLANT PHOSPHOLIPIDS, GLYCOLIPIDS, AND CHLOROPHYLL

Concentrations in (moles/l) × 10^4

Source	*P-lipids*						*Chlorophyll*	*Glycolipids*		
	GPI	*GPG*	*GPE*	*GPC*	*GP*	*Total*		*G-gal-SO$_3$H*	*G-gal-gal*	*G-gal*
Spinach, leaves	2	6	4	11	0.5	23	11			
Spinach, chloroplasts	6	24	3	19	1	53	105			
Beet, leaves	5	9	11	22	0	47	14	9	13	24
Beet, chloroplasts	6	23	9	24	7*	69	72	15	68	121
Elder, green	3	17	5	19	0	44	28	5	26	56
Elder, yellow	4	7	5	18	0	33	2.5	4	11	17
Bean seedlings						Unknowns				
0.5 h light daily	3.5	5.5	6	15	5	35	1	2	6	10
2 h light daily	3.5	7	6.5	11.5	5	33.5	6	4	9	13
8 h light daily	3.5	9.5	6	13	3	35	27	2	14	39

* Since GP was observed in the chloroplasts, but not in the whole leaves, it must be an artifact, arising during the preparation of the chloroplasts. See for chloroplast lecithinase C: KATES[21]. In the data of Table II, a correction has been made for this partial hydrolysis. This does not, however, substantially influence the results and the conclusions.

[§] Leaf volume is estimated from fresh weight, assuming an average specific weight = 1.

TABLE II

PHOSPHOLIPIDS AND GLYCOLIPIDS:
AMOUNTS PER UNIT VOLUME OF FRESH LEAVES, EXPRESSED AS (MOLES/l) × 10^4

	GPI	*GPG*	*GPE*	*GPC*	*Total P*	*G-gal-SO$_3$H*	*G-gal-gal*	*G-gal*
Spinach, total leaf:	2.45	5.2	2.8	11.05	21.5			
(1) chloroplasts (10% of leaf volume)	0.75	2.6	0.1	2.2	5.65			
(2) cytoplasm (by difference)	1.7	2.6	2.7	8.85	15.85			
Percent in chloroplasts:	30	50	3	20	26			
Beet, total leaf:	4.8	8.7	10.8	22.5	47	9	13	24
(1) chloroplasts (20% of leaf volume)	1.3	5.2	2.1	5.2	14	3	13.5	24
(2) cytoplasm (by difference)	3.5	3.5	8.7	17.3	33	6	0	0
Percent in chloroplasts:	27	60	20	23	30	33	100	100

pared with total cellular phosphatides, and glycolipids. The actual concentrations in cytoplasm cannot be estimated directly, without reliable data on the volume of the vacuoles.

The averaged results from those experiments in which the leaf- and chloroplast lipids were analysed from a single batch of material are given in Table II. The distribution of phosphatide-P among the 4 major fractions, in chloroplasts and cytoplasm, is visible in Table III.

TABLE III

PHOSPHATIDE COMPOSITION OF CHLOROPLASTS AND CYTOPLASM

Source	*% phosphatidyl ester*			
	GPI	*GPG*	*GPE*	*GPC*
Spinach: chloroplasts	13	46	2	39
cytoplasm	11	16	17	56
Beet: chloroplasts	9	38	15	38
cytoplasm	11	11	26	52

Phosphatides

The high percentage of GPG-lipid in the chloroplasts is again evident from comparison of the concentrations in leaf *vs.* chloroplasts in the two species *Spinacia* and *Beta*.

The data of Table II show that the chloroplasts are not particularly rich in phosphatides. For spinach leaves MENKE AND JAKOB[10] reported that the chloroplasts contain about 35 % of total leaf protein, and about 18 % of the dry weight. Taking into consideration that the cell walls constitute an important fraction of the dry weight —26.5 % for spinach leaves[10]—, we calculate that the chloroplasts contain about 25 %

of the cell contents and we must conclude that the ratios of phosphatide : protein or phosphatide : dry weight of cell contents, for chloroplasts and other cell contents are not significantly different. (On the other hand, it is well proved that the chloroplasts are particularly rich in lipoid matter (see ref. 11 p. 372).) The composition of the phosphatides is quite different, however, in chloroplasts and other cell contents, as appears from Table III. Chloroplasts are particularly rich in GPG-lipid, as was concluded already from earlier experiments[3]. The remainder of the cell has a much higher percentage of the classical, N-containing phosphatides, lecithin and cephalin.

In the experiments with green *vs.* yellow leaves, the same trend is observed, both in the damaged leaves of *Sambucus* and in the immature leaves of *Phaseolus*. In *Sambucus*, the total phosphatide-P is significantly lower in the yellow leaves; this difference is entirely due to the lack of phosphatidyl glycerol. With the beans of different degrees of greening, the only important difference is also in the GPG-lipid.

Some unknown compounds have a certain importance in *Phaseolus*. Judging from the R_F values, among the observed spots may be GPS and GPGPG, the deacylation products of, respectively, phosphatidyl serine, and the recently discovered diphosphatidyl glycerol[12]. Unknown spots, similar to those seen in the *Phaseolus* experiments have also been observed with *Xanthium* leaves and with *Lemna*.

From theoretical considerations regarding the structure of the chloroplasts, importance has been attached to the molar ratio of chlorophyll : phosphatides; see for a discussion ref. 11, p. 391 and p. 1742. From data by GODNEV *et al.*[13], a molar ratio can be calculated: 5.0 for rye, and 2.2 for lettuce. From the present experiments the ratio for spinach chloroplasts is calculated: 2.0, and for beet chloroplasts: 1.05. Incidentally, the latter value closely approaches the "theoretical" value, postulated by HUBERT's theory[14] (see also ref. 11).

Glycolipids and sulfolipid

Since analyses of glycolipids and sulfolipid are still scarce, it seems worthwile to give some data regarding quantities involved.

The method of determination used, has been reported to be equally sensitive for mono-, di- and polysaccharides[9,15]. Hence, it can be assumed that acid-labile linkages do not interfere, and the measured extinctions have been evaluated by comparison with a galactose standard. However, the carbohydrate-sulfonate linkage in the sulfolipid is acid-stable[16], and the results regarding the G-gal-SO_3 would be more accurate, if an authentic sample had been available. Indeed, the time course of colour development with the anthrone reagent was somewhat slower in this case, than with the galactosyl-glycerols and the galactose standard. By comparison of the relative size and intensity of the spots (see Fig. 1), however, the distribution of sugar over the three compounds investigated seems to agree reasonably with the chemical data of Table I.

For the galactolipids, the concentrations measured indicate that they are major lipids of the green leaves. Moreover, the results with beet leaves and chloroplasts (Table II) show that the galactolipids are found specifically in the chloroplasts, where they are found in concentrations up to, and even exceeding 10^{-2} *M*, in agreement with earlier estimates[3]. In the experiments with *Sambucus* and *Phaseolus*, the concentrations in the green leaves were significantly higher than in the yellow ones. The fact that there are still important amounts of galactolipids, even when chlorophyll concentrations are very low, does not prove that these lipids occur in the cytoplasm, since there may be a more or less developed plastid system. This seems supported by the recent observation that one short illumination permitted development of the plastids of dark-grown *Phaseolus vulgaris* to near-normal size, without concomitant formation of chlorophyll[17].

The sulfolipid, in our experiments, seemed less specifically connected with the photosynthetic apparatus. In the experiments with green *vs.* yellow leaves, the concentrations of this compound were not significantly different at different chlorophyll concentrations. In the beet experiments, it appeared that the compound occurred both in the cytoplasm and in the chloroplasts.

In connection with this publication, it is interesting to draw attention to a series of investigations on plant phosphatides by WINTERSTEIN, HIESTAND and coworkers, now over 50 years ago[18, 19]. From various plant materials, they isolated lipid fractions which were submitted to acid hydrolysis. Apart from P and N in varying percentages and ratios, important amounts of reducing sugars were found. Maximum reducing power was only liberated after prolonged hydrolysis, from which it was concluded that chemical binding, rather than adsorption or coprecipitation had to be assumed. Galactose was isolated from the hydrolyzate in measurable amounts, and also glucose and pentose were demonstrated. It seems very probable that the galactolipids were involved. The highest % of sugar was found in lipids from wheat flour—the same source from which the galactolipids were isolated by CARTER *et al.*[20].

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REFERENCES

[1] A. A. BENSON AND B. MARUO, *Biochim. Biophys. Acta*, 27 (1958) 189.
[2] A. A. BENSON, R. WISER, R. A. FERRARI AND J. A. MILLER, *J. Am. Chem. Soc.*, 80 (1958) 4740.
[3] A. A. BENSON, J. F. G. M. WINTERMANS AND R. WISER, *Plant Physiol.*, 34 (1959) 315.
[4] A. T. JAGENDORF AND M. AVRON, *J. Biol. Chem.*, 231 (1958) 277.
[5] R. HILL AND D. A. WALKER, *Plant Physiol.*, 34 (1959) 240.
[6] E. HEIERLE, *Ber. schweiz. botan. Ges.*, 44 (1935) 17.
[7] C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
[8] J. G. BUCHANAN, G. A. DEKKER AND A. G. LANG, *J. Chem. Soc.*, (1950) 3162.
[9] E. W. YEMM AND A. J. WILLIS, *Biol. J.*, 57 (1934) 508.
[10] W. MENKE AND E. JAKOB, *Z. physiol. Chem. Hoppe Seyler's*, 272 (1942) 227.
[11] E. RABINOWITCH, *Photosynthesis* I; II, 2, Interscience Publ.
[12] A. A. BENSON AND E. H. STRICKLAND, *Biochim. Biophys. Acta*, 41 (1960) 328.
[13] T. N. GODNEV, A. A. SHLIK AND N. K. TRETJAK, *Doklady Akad. Nauk SSSR*, 87 (1952) 493.
[14] B. HUBERT, *Rec. trav. botan. néerl.*, 32 (1935) 323.
[15] R. M. MCGREADY, J. GUGGOLS, V. SILVIERA AND H. S. OWENS, *Anal. Chem.*, 22 (1950) 1156.
[16] A. A. BENSON, H. DANIEL AND R. WISER, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 1582.
[17] J. MEGO AND A. T. JAGENDORF, *Plant Physiol.*, 34 (1959) suppl. xix.
[18] E. WINTERSTEIN AND O. HIESTAND, *Z. physiol. Chem. Hoppe Seyler's*, 54 (1908) 288.
[19] E. WINTERSTEIN AND K. SMOLENSKI, *Z. physiol. Chem. Hoppe Seyler's*, 58 (1909) 506.
[20] H. E. CARTER, R. H. MCCLUER AND E. C. SLIFER, *J. Am. Chem. Soc.*, 78 (1956) 3735.
[21] M. KATES, *Can. J. Biochem. and Physiol.*, 34 (1956) 967.

ACTIVATION DES ACIDES AMINÉS PAR LES MICROSOMES ET L'ENZYME pH 5 DE RÉTICULOCYTES DE LAPIN

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SUMMARY

Activation of amino acids by the microsomes and the pH-5-enzyme of rabbit reticulocytes

Microsomes from reticulocytes are able to activate aminoacids, as it is established by the measurement of the increase of the amino acid dependent ATP–^{32}PP exchanges. The activating properties of the microsomes are compared to those of the pH-5-enzyme from rabbit reticulocytes. They are similar, but not identical, and not additive.

INTRODUCTION

L'incorporation des acides aminés dans les protéines est précédée par l'activation de leur groupement carboxyl. Cette activation met en jeu l'ATP, l'ion Mg^{++}, et des enzymes spécifiques; ceux-ci se trouvent dans le surnageant de l'ultracentrifugation après sédimentation de toutes les particules. On peut les précipiter en amenant le milieu au pH 5.2, comme l'ont mis en évidence ZAMECNIK, HOAGLAND et leurs collaborateurs[1,2] qui ont appelé cette préparation "enzyme pH 5". La mise en évidence de l'activation des acides aminés se fait en particulier grâce à la réaction réversible suivante[3,4], E étant l'enzyme activateur:

$$ATP + AA + E \leftrightharpoons AMP\text{–}AA\text{–}E + PP$$

L'activation se mesure en incubant la préparation enzymatique avec l'acide aminé, ^{32}PP, ATP et Mg^{++} puis en isolant l'ATP sur charbon et en mesurant son activité spécifique.

Cependant, récemment des enzymes activateurs ont été mis en évidence dans les mitochondries de cellules hépatiques. REIS *et al.*[5] d'une part, CRADDOCK ET SIMPSON[6] d'autre part on extrait de ces particules une fraction se comportant comme l'enzyme pH 5, mais les mitochondries entières ne possèdent pas cette propriété activatrice. Par ailleurs, COHN[7] a isolé des microsomes de cellules de foie de rat une fraction qui incorpore les acides aminés en absence d'enzyme pH 5; cependant les enzymes activateurs n'ont pu être mis en évidence dans cette fraction. Dans les cellules végé-

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Abréviations: AA, acide aminé; ATP, adénosine triphosphate; AMP, adénosine monophosphate; PP, pyrophosphate; Tris, tris(hydroxyméthyl)aminométhane.

tales, WEBSTER[8] a pu isoler à partir de particules ribonucléoprotéiques une fraction jouant le rôle d'enzyme pH 5. Par ailleurs, SCHWEET *et al.*[9] ont observé une incorporation d'acides aminés dans les microsomes et l'hémoglobine de système acellulaire provenant de réticulocytes de lapin. Cette incorporation se fait en présence d'enzyme pH 5, il est cependant remarquable qu'en absence de cet enzyme, l'incorporation, quoique fortement abaissée, soit loin d'être négligeable. Ceci nous a amené à chercher un rôle activateur possible des microsomes de réticulocytes.

MÉTHODES

Obtention des réticulocytes

Les lapins reçoivent 5 injections sous-cutanées, à raison d'une par jour, de solution neutralisée de chlorhydrate de phénylhydrazine à 2.5 %, à la dose de 0.4 ml/kg de poids et par injection. Le sang est prélevé par ponction cardiaque, les globules rouges sont centrifugés et lavés deux fois avec du sérum physiologique.

Préparation des fractions réticulocytaires

On opère selon la technique de SCHWEET *et al.*[9]. Les globules rouges lavés sont hémolysés à froid par addition de 4 volumes d'une solution de chlorure de magnésium 0.005 M et agitation magnétique pendant 10 min. On ajoute ensuite lentement 1 volume d'une solution formée de saccharose 1.5 M et de chlorure de potassium 0.15 M et on centrifuge 10 min à 10,000 × g, le surnageant est centrifugé ensuite 60 min à 105,000 × g dans une centrifugeuse Spinco préparative.

Le précipité est lavé par la solution de chlorure de magnésium pour éliminer toute trace de surnageant. Dans quelques expériences les microsomes sont remis en suspension dans un mélange formé de 4 volumes de la solution de chlorure de magnésium et 1 volume de la solution de saccharose–KCl, puis centrifugés de nouveau 60 min à 105,000 × g. Certaines expériences ont été réalisées, non avec des microsomes entiers, mais avec la fraction insoluble dans le désoxycholate de sodium, selon LITTLEFIELD *et al.*[10]. Pour cela les microsomes sont mis en suspension dans une solution froide préparée extemporanément de désoxycholate de sodium à 5 % dans un tampon Tris à pH 8.0, le poids de désoxycholate représentant les trois quarts de celui des microsomes. Après addition du mélange formé de 4 volumes de la solution de chlorure de magnésium et de 1 volume de la solution de saccharose–KCl, la préparation est centrifugée de nouveau 60 min à 105,000 × g. Dans tous les cas les microsomes sont mis en suspension dans 2 ml de tampon Tris à pH 7.8 et dialysés 16 heures contre le même tampon. Le surnageant de la première centrifugation rapide est amené à pH 5.15 avec de l'acide acétique 1.0 N; le précipité est centrifugé, lavé pour éliminer l'hémoglobine, puis dissous dans 2 ml de tampon Tris pH 7.8 et dialysé 16 h contre le même tampon. La concentration en protéine est déterminée par le réactif du biuret.

Incubation

Le milieu d'incubation est voisin de celui décrit par DEMOSS ET NOVELLI[4]. Sauf indication contraire dans certaines expériences, on mélange dans un petit tube à centrifuger: 10 μmoles KF, 10 μmoles ATP neutralisé, 10 μmoles ^{32}PP contenant entre 60 et 150,000 coups/min, la préparation enzymatique, 2 μmoles d'acide aminé, 10 μmoles $MgCl_2$, 100 μmoles tampon Tris pH 7.8. Le volume total est de 1 ml, on incube en général 30 min dans un incubateur de Dubnoff avec agitation à 37°. La réaction est arrêtée par addition de 1 ml d'acide trichloroacétique à 5 %.

Préparation de l'ATP. Mesure de la radioactivité

Après centrifugation, le surnageant de la précipitation trichloroacétique est décanté dans un tube à centrifuger contenant 100 mg de charbon Norit A, selon la technique de CRANE ET LIPMANN[11], on agite et on centrifuge, le charbon est lavé 4 fois par mise en suspension dans 10 ml d'eau et centrifugation pour éliminer les dérivés phosphorés minéraux. Le charbon est ensuite mis en suspension homogène dans 2 ml d'une solution d'ammoniaque 0.3 M dans de l'alcool à 50 %. Une partie aliquote est transférée dans une cupule de cuivre de 2.85 cm^2 dans laquelle on a fixé une rondelle de papier mince; elle est desséchée sous infra-rouge et pesée, la radioactivité est mesurée au compteur de Geiger-Müller à fenêtre mince. Après correction d'épaisseur, on calcule la radioactivité correspondant à 100 mg de charbon.

Le nombre de μmoles de ^{32}PP incorporé dans l'ATP est donné par la formule suivante, sachant que l'incubation a été faite en présence de 10 μmoles de PP et 10 μmoles d'ATP:

$$\frac{\text{Activité spécifique ATP}}{\text{Activité spécifique PP}} \times 10.$$

On détermine également le pourcentage d'échange selon DEMOSS ET NOVELLI[4]:

$$\frac{\text{Activité spécifique ATP}}{0.5 \times \text{Activité spécifique PP}} \times 100.$$

RÉSULTATS

Activation des différents acides aminés (Tableau I)

L'incubation se fait en présence soit de microsomes soit d'enzyme pH 5 en quantité correspondant à 2 mg de protéine, de 5 μmoles de chacun des acides aminés, de forme L. Elle dure 30 min. On voit que les acides aminés suivants augmentent au moins 2 fois les échanges ATP–PP, par ordre d'activation décroissante:

Microsomes: méthionine, valine, phénylalanine, tyrosine, leucine, glutamine, lysine, acide aspartique, asparagine, isoleucine, arginine et acide glutamique.

Enzyme pH 5: méthionine, lysine, isoleucine, leucine, valine, tyrosine, glutamine, alanine, cystéine, asparagine, arginine, acide glutamique et histidine.

Dans l'ensemble, ce sont les mêmes acides aminés qui augmentent les échanges ATP–PP avec les deux systèmes, à l'exception toutefois de la phénylalanine qui n'est pas activée par l'enzyme pH 5, et l'est par les microsomes ainsi que l'analogue de cet acide aminé, la fluorophénylalanine. Il a été montré précédemment que cet analogue peut être incorporé dans l'hémoglobine des réticulocytes[12]. Par contre la norleucine n'est pas activée.

Additivité des activations de différents acides aminés (Tableau II)

L'incubation se fait en présence soit de microsomes, soit d'enzyme pH 5 en

TABLEAU I

ACTIVATION DES DIFFÉRENTS ACIDES AMINÉS PAR LES MICROSOMES ET L'ENZYME pH 5

Le milieu contenait: 10 μmoles ATP, 10 μmoles ^{32}PP (15,000 coups/min/μmole), 5 μmoles AA, 10 μmoles $MgCl_2$, 10 μmoles KF, 100 μmoles tampon Tris pH 7.8. Enzyme pH 5 et microsomes correspondant à 2 mg protéine, 30 min incubation à 37°.

Acides aminés ajoutés au milieu	*Microsomes*			*Enzyme pH 5*		
	μmoles PP incorporé	*Pour cent échanges PP–ATP*	*Augmentation des échanges*	*μmoles PP incorporé*	*Pour cent échanges PP–ATP*	*Augmentation des échanges*
Sans acide aminé	0.57	11.4		0.62	12.4	
L-méthionine	3.62	72.4	6.35	2.96	59.8	4.82
L-valine	2.80	56.0	4.90	2.12	42.4	3.42
L-phenylalanine	2.67	53.4	4.70	1.05	21.0	1.80
L-tyrosine	1.92	38.4	3.36	2.70	41.0	3.30
L-leucine	1.74	34.8	3.06	2.20	44.0	3.55
Glutamine	1.73	34.6	3.03	1.92	38.4	3.10
L-lysine	1.72	34.4	3.02	2.36	47.2	3.80
Acide L-aspartique	1.40	28.0	3.26	1.89	37.8	3.04
Asparagine	1.27	25.4	2.23	1.78	35.6	2.87
L-cystéine	1.17	23.4	2.05	1.86	37.2	3.00
L-isoleucine	1.16	23.2	2.04	2.18	43.6	3.62
L-arginine	1.14	22.8	2.00	1.68	33.6	2.70
Acide L-glutamique	1.14	22.4	2.00	1.40	28.0	2.26
L-histidine	0.93	18.6	1.63	1.28	25.6	2.06
L-thréonine	0.90	18.0	1.58	1.20	24.0	1.93
L-tryptophane	0.75	15.0	1.31	0.74	14.8	1.19
L-alanine	0.66	13.2	1.16	1.90	38.0	3.06
L-proline	0.58	11.6	1.02	0.85	17.0	1.37
L-sérine	0.57	11.4	1.00	1.02	20.4	1.65
Glycocolle	0.57	11.4	1.00	0.75	15.0	1.21
Fluorophénylalanine	2.13	42.6	3.74	0.70	12.4	1.13
Norleucine	0.59	11.8	1.04	0.70	12.4	1.13

TABLEAU II

ADDITIVITÉ DES ACTIVATIONS DE DIFFÉRENTS ACIDES AMINÉS

Le milieu contenait: 10 μmoles ATP, 10 μmoles ^{32}PP (11,500 coups/min/μmole), 2 μmoles AA, 10 μmoles $MgCl_2$, 10 μmoles KF, 100 μmoles tampon Tris pH 7.8. Enzyme pH 5 et microsomes correspondant à 1 mg protéine, 30 min incubation à 37°.

Acides amines ajoutés au milieu	*Microsomes*			*Enzyme pH 5*		
	μmoles PP incorporé	*Pour cent échanges PP–ATP*	*Augmentation des échanges*	*μmoles PP incorporé*	*Pour cent échanges PP–ATP*	*Augmentation des échanges*
Sans acide aminé	0.30	6.0		0.20	4.0	
+ L-valine	1.47	29.4	4.9	0.70	14.0	3.5
+ L-lysine	0.92	18.4	3.1	0.89	17.8	4.35
+ L-leucine	1.00	20.0	3.34	0.65	13.0	3.25
+ L-valine + L-lysine	2.30	46	7.60	1.52	30.4	7.65
+ L-valine + L-lysine + L-leucine	3.30	66.0	11	2.15	43	10.7

quantité correspondant à 1.0 mg de protéine et de 2 μmoles d'acide aminé. On constate avec les trois acides aminés étudiés qu'il y a additivité des échanges ATP-PP provoqués par chacun des acides aminés.

TABLEAU III

NON ADDITIVITÉ DES ACTIVATIONS PAR L'ENZYME pH 5 ET LES MICROSOMES

Le milieu contenait: 10 μmoles ATP, 10 μmoles ^{32}PP (9,000 coups/min/μmole), 2 μmoles AA, 10 μmoles $MgCl_2$, 10 μmoles KF, 100 μmoles tampon Tris pH 7.8, 30 min incubation à 37°.

	Sans acide aminé		*2 μmoles L-valine*			*2 μmoles L-tyrosine*		
	μmoles PP incorporé	*Pour cent échanges ATP–PP*	*μmoles PP incorporé*	*Pour cent échanges ATP–PP*	*Augmentation des échanges*	*μmoles PP incorporé*	*Pour cent échanges ATP–PP*	*Augmentation des échanges*
Microsomes: 2 mg protéines	0.50	10.0	1.96	39.2	3.92	1.76	35.2	3.52
Microsomes: 4 mg protéines	0.66	13.2	2.58	51.6	3.90	2.07	41.4	3.13
Enzyme pH 5: 2 mg protéines	0.63	12.7	2.54	50.8	4.0	2.44	48.8	3.85
Enzyme pH 5: 4 mg protéines	0.89	17.8	2.95	59.0	3.32	2.77	55.4	3.10
Microsomes: 2 mg protéines + Enzyme pH 5: 2 mg protéines	0.80	16.0	2.90	58.0	3.63	2.53	50.6	3.17

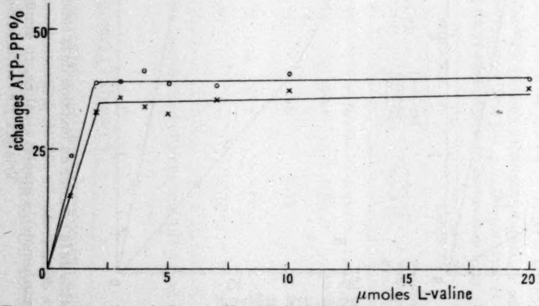

Fig. 1. Activation de la L-valine en fonction de sa concentration: O—O, en présence de microsomes; ×—×, en présence d'enzyme pH 5. 10 μmoles ATP, 10 μmoles PP (7,550 coups/min/μmole), 10 μmoles KF, 10 μmoles $MgCl_2$, 100 μmoles tampon Tris pH 7.8. Microsomes et enzyme pH 5 correspondant à 1 mg protéine. Incubation 30 min à 37°.

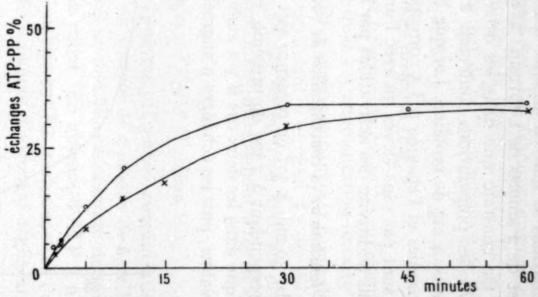

Fig. 2. Activation de la L-valine en fonction de la durée de l'incubation: O—O, en présence de microsomes; ×—×, en présence d'enzyme pH 5. 10 μmoles ATP, 10 μmoles ^{32}PP (6,250 coups/min/μmole), 2 μmoles L-valine, 10 μmoles KF, 10 μmoles $MgCl_2$, 100 μmoles tampon Tris pH 7.8. Microsomes et enzyme pH 5 correspondant à 1 mg protéine. Incubation à 37°.

Non additivité des activations par les microsomes et l'enzyme pH 5 (Tableau III)

Les incubations se font en absence d'acide aminé et en présence de 2 µmoles de L-valine et de 2 µmoles de L-tyrosine avec les deux préparations enzymatiques séparément. On constate que dans les deux cas l'augmentation des échanges est maximale avec les préparations contenant 2 mg de protéine, puisqu'elle n'est pas plus élevée avec 4 mg de protéine. Lorsque l'on incube ces acides aminés avec à la fois les microsomes et l'enzyme pH 5 correspondant chacun à 2 mg de protéine, les échanges ne sont pas plus élevés qu'avec l'une des deux préparations seulement. Il n'y a donc pas additivité des activations par les microsomes et l'enzyme pH 5.

Activation en fonction de la concentration de valine (Fig. 1)

On incube 30 min à 37° en présence soit de microsomes soit d'enzyme pH 5 en quantité correspondant à 1 mg de protéine, et de quantités croissantes de L-valine. On constate que dans les deux cas il y a augmentation linéaire des échanges jusqu'à 2 µmoles de valine, puis les échanges n'augmentent plus jusqu'à 20 µmoles de l'acide aminé.

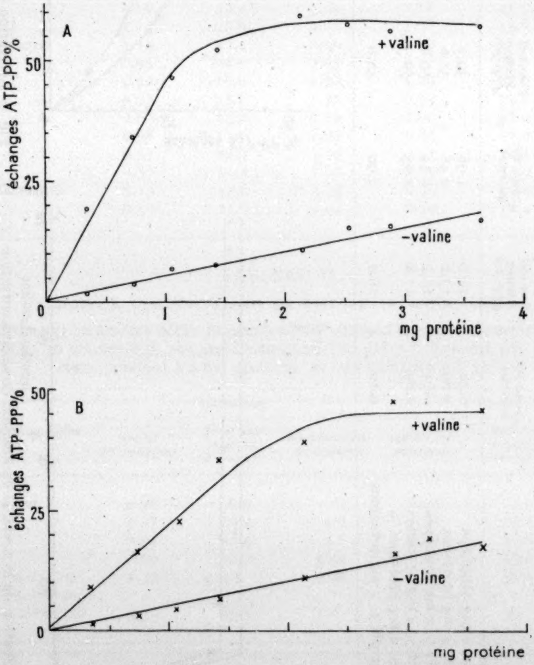

Fig. 3. Activation de la L-valine en fonction de la concentration en microsomes et en enzyme pH 5: A, en présence de microsomes; B, en présence d'enzyme pH 5. 10 µmoles ATP, 10 µmoles ^{32}PP (8,550 coups/min/µmole), 2 µmoles L-valine, 10 µmoles KF, 10 µmoles $MgCl_2$, 100 µmoles tampon Tris pH 7.8. Incubation 30 min à 37°.

Activation en fonction de la durée de l'incubation (Fig. 2)

On incube 2 µmoles de L-valine en présence soit de microsomes soit d'enzyme pH 5 correspondant à 1 mg de protéine à 37°. Les échanges augmentent jusqu'à la 30ème minute, puis restent constants jusqu'à la 60ème minute. Entre la 115ème et la 130ème minutes les échanges augmentent de 60 % en présence de l'enzyme pH 5 et de 30 % en présence de microsomes.

Activation en fonction de la concentration en protéines (Fig. 3)

On incube 2 µmoles de L-valine en présence de quantités croissantes de microsomes et d'enzyme pH 5. On constate qu'il y a augmentation linéaire des échanges jusqu'à une concentration d'enzyme pH 5 correspondant à 1.6 mg de protéine et une concentration de microsomes correspondant à environ 1.1 mg de protéine. Les échanges restent constants entre 2 et 3.5 mg de protéine avec les deux systèmes. En absence de valine les échanges augmentent linéairement avec la concentration en protéine.

Action de quelques facteurs sur les échanges ATP-PP (Tableau IV)

On incube soit les microsomes soit l'enzyme pH 5 à une concentration correspondant à 1 mg de protéine en présence et en absence de 2 µmoles de L-valine. La valine augmente les échanges de 3.6 fois. Si on ne met pas de chlorure de magnésium, les échanges ne sont pas augmentés par la valine. En absence d'ATP il n'y a que très peu de radioactivité. Si on chauffe préalablement les microsomes et l'enzyme pH 5 au bain-marie à 100° pendant 10 min les échanges deviennent négligeables.

TABLEAU IV

ACTION DE DIFFÉRENTS FACTEURS SUR L'ACTIVATION DE LA L-VALINE

Le milieu contenait: 10 µmoles ATP, 10 µmoles ^{32}PP (7,500 coups/min/µmoles), 2 µmoles L-valine, 10 µmoles $MgCl_2$, 10 µmoles KF, 100 µmoles tampon Tris pH 7.8. Microsomes et enzyme pH 5 correspondant chacun à 1 mg de protéine, 30 min incubation à 37°.

	Microsomes		*Enzyme pH 5*	
	µmoles PP incorporé	*Pour cent échanges PP–ATP*	*µmoles PP incorporé*	*Pour cent échanges PP–ATP*
Complet — acide aminé	0.58	11.6	0.52	10.3
Complet + L-valine	2.12	42.4	1.80	36.0
Complet + L-valine — ATP	0.14	2.9	0.13	2.60
Complet + L-valine — Mg^{++}	0.62	12.4	0.60	12.0
Complet + L-valine protéines chauffées*	0.06	1.2	0.03	0.6

* Avant l'addition des autres composés les microsomes et l'enzyme pH 5 sont placés 10 min dans un bain-marie à 100°.

Purification et fractionnement des microsomes (Tableau V)

Pour s'assurer qu'il n'y a aucune contamination des microsomes par des enzymes du surnageant, on lave les microsomes (voir MÉTHODES) et on incube une quantité correspondant à 1 mg de protéine avec 2 µmoles de L-valine. On constate que les échanges sont sensiblement les mêmes que l'on incube avec les microsomes préparés selon la méthode habituelle ou avec les microsomes purifiés, mais dans ce dernier cas les échanges sont fortement abaissés en absence de valine, si bien que l'augmentation des échanges due à la valine est de 11.0 fois avec les microsomes purifiés au lieu de 2.8 fois avec les microsomes préparés par la méthode habituelle. Avec la fraction de microsome insoluble dans le désoxycholate il y a à la fois abaissement des échanges en présence et en absence de valine. L'augmentation des échanges due à la valine est dans ce cas de 10.5 fois.

TABLEAU V

ACTIVATION DE LA L-VALINE PAR DIFFÉRENTES PRÉPARATIONS DE MICROSOMES

Le milieu contenait: 10 µmoles ATP, 10 µmoles ^{32}PP (6,000 coups/min/µmole), 2 µmoles L-valine, 10 µmoles $MgCl_2$, 10 µmoles KF, 100 µmoles tampon Tris pH 7.8. Microsomes correspondant à 1 mg de protéine.

	Sans valine		*Avec valine*		
	µmoles PP incorporé	*Pour cent échanges PP–ATP*	*µmoles PP incorporé*	*Pour cent échanges PP–ATP*	*Augmentation des échanges*
Microsomes	0.70	14	1.97	39.4	2.80
Microsomes lavés*	0.16	3.2	1.77	35.4	11.0
Fraction microsomique insoluble dans desoxycholate**	0.10	2.0	1.05	21.0	10.5

* Les microsomes sont remis en suspension dans 10 ml de solution $MgCl_2$–saccharose–KCl et centrifugés une 2ème fois 60 min à 105,000 × *g*.

** Les microsomes sont traités par le désoxycholate de sodium selon la technique de LITTLEFIELD *et al.*[10] et centrifugés une 2ème fois 60 min à 105,000 × *g*.

DISCUSSION

Le schéma classique de HOAGLAND de l'activation des acides aminés et de leur incorporation dans les protéines peut s'appliquer aux réticulocytes comme l'ont montré SCHWEET *et al.*[9]. Cependant les résultats décrits ici montrent que l'enzyme pH 5 n'est pas la seule fraction cellulaire capable d'activer les acides aminés, cette activation pouvant être réalisée par les microsomes. La première question qui se pose est de savoir si cette activation par les microsomes n'est pas en réalité due à une contamination par le surnageant. La purification des microsomes qui ne diminue pas le pouvoir activateur permet d'éliminer formellement cette possibilité. La deuxième question est de savoir si les deux systèmes sont identiques. Il est plus difficile d'y répondre. Les facteurs cinétiques sont très voisins. Les différences peuvent être dues aux différences d'environnement. Dans les deux cas l'ion Mg^{++} est indispensable, le chauffage abolit toute activité. Il n'y a pas identité de l'activation des acides aminés individuels par les deux systèmes, en particulier la phénylalanine et la fluorophénylalanine ne sont activées que par les microsomes. L'alanine n'est activée que par l'enzyme pH 5; il n'est cependant pas exclu que l'enzyme pH 5 ne contient pas la totalité des enzymes activateurs du surnageant. Le mécanisme de l'activation par l'enzyme pH 5 et les microsomes semble identique, puisque leur action n'est pas additive. Le fait qu'en absence d'enzyme pH 5 l'incorporation des acides aminés dans les microsomes de systèmes acellulaires de microsomes soit faible, peut s'expliquer par la seule absence dans le milieu d'acide ribonucléique soluble. Des expériences sont entreprises pour tester cette hypothèse. Le rôle physiologique des propriétés activatrices des microsomes est difficile à comprendre actuellement. Les microsomes complètent-ils simplement l'action de la fraction non particulaire ou bien possèdent-ils un rôle particulier dans la synthèse protéique en plus de ceux qui leur sont actuellement attribués?

RÉSUMÉ

Les microsomes de réticulocytes sont capables d'activer les acides aminés comme on peut l'établir par l'augmentation des échanges ATP–^{32}PP.

Les propriétés activatrices des microsomes et de l'enzyme pH 5 ont été comparées: elles sont voisines quoique non identiques et elles ne sont pas additives.

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BIBLIOGRAPHIE

[1] P. C. ZAMECNIK ET E. B. KELLER, *J. Biol. Chem.*, 209 (1954) 337.
[2] M. B. HOAGLAND, E. B. KELLER ET P. C. ZAMECNIK, *J. Biol. Chem.*, 218 (1956) 345.
[3] M. B. HOAGLAND, *Biochim. Biophys. Acta*, 16 (1955) 288.
[4] J. A. DEMOSS ET G. D. NOVELLI, *Biochim. Biophys. Acta*, 22 (1956) 49.
[5] P. R. REISS, J. L. COOTE ET T. S. WORK, *Biochem. J.*, 72 (1959) 24.
[6] V. M. CRADDOCK ET M. V. SIMPSON, *Biochem. J.*, 74 (1960) 10 P.
[7] P. COHN, *Biochem. J.*, 73 (1959) 25 P.
[8] G. C. WEBSTER, *J. Biol. Chem.*, 229 (1957) 535.
[9] R. S. SCHWEET, H. LAMFROM ET E. ALLEN, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 1029.
[10] J. W. LITTLEFIELD, E. B. KELLER, J. GROSS ET P. C. ZAMECNIK, *J. Biol. Chem.*, 217 (1955) 111.
[11] R. K. CRANE ET F. LIPMANN, *J. Biol. Chem.*, 201 (1953) 235.
[12] J. KRUH ET J. ROSA, *Biochim. Biophys. Acta*, 34 (1959) 561.

IN VITRO CONVERSION OF PTEROYLGLUTAMIC ACID TO CITROVORUM FACTOR BY RAT LIVER ENZYMES

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SUMMARY

The enzymes in rat liver which convert pteroylglutamic acid to citrovorum factor are found to reside in the mitochondria and are dissociable into two fractions, one bringing about the initial reduction of pteroylglutamic acid to dihydropteroylglutamic acid and the other effecting the final reduction to tetrahydropteroylglutamic acid followed by formylation to the citrovorum factor-active derivatives; the former is easily released into the supernatant in isotonic saline, while the latter is bound more firmly in the mitochondrion.

The enzyme complex has been partially purified from 0.05 % sodium desoxycholate mitochondrial extract by precipitation at 30–50 % $(NH_4)_2SO_4$ saturation.

Reducing agents act as non-specific stabilizers and a nitrogen atmosphere is better for CF synthesis when compared to ascorbic acid addition. Serine is the most effective formyl donor. TPNH, ATP, Mg^{++}, homocysteine and pyridoxal phosphate are important, in that order, for optimal conversion. Their role in the conversion mechanism has been discussed.

INTRODUCTION

There have already been reports relating to the enzymic conversion of PGA to CF by bacterial[1], rat and chicken liver[2,3] preparations. Anaerobic conditions[4] as well as a source of formate[1–3] have been shown to be essential for optimal conversion. DHPGA[5,6] and THPGA[7,8] have been shown to be intermediates preceding the formylated active and labile THPGA derivatives which yield stable CF after anaerobic and non-enzymic degradation[9]. The present report relates to the CF-synthesising system from rat liver preparations.

METHODS

Animals

Albino rats (Wistar strain), 100–120 g in weight and of either sex were used. They were maintained on a laboratory stock diet which consisted of 75 % whole

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Abbreviations: PGA, pteroylglutamic acid; CF, citrovorum factor; N^5, formyl tetrahydropteroylglutamic acid; DHPGA, dihydropteroylglutamic acid; THPGA, tetrahydropteroylglutamic acid; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.

wheat flour, 2 % whole milk powder, 12 % casein, 2 % dried yeast, 4 % arachis oil, 1 % vitaminised sesame oil (2000 I.U. of vitamin A and 50 I.U. of vitamin D per kg diet), 2 % sodium chloride and 2 % calcium carbonate.

Preparation of cell fractions

The animals were stunned by a blow on the head, exsanguinated and the liver removed and chilled in crushed ice. Portions of the liver were made up to 20 % homogenates in isotonic sucrose and fractionated using an International Refrigerated Centrifuge (Model PR-2) to yield the nuclear, mitochondrial and supernatant fractions according to the method of HOGEBOOM AND SCHNEIDER[10]. The supernatant was not centrifuged in order to separate the microsomes. When isotonic saline was used as the medium for homogenation, fractions were collected at 700 × *g* for 10 min (Fraction 700 × *g*) and further at 5000 × *g* for 10 min (Fraction 5000 × *g*). The particulate fractions were washed by resuspension in isotonic saline and fractionation using the same speeds and times.

Partial purification of the enzymes

For solubilizing the enzyme complex, mitochondria isolated from 5 *g* liver[10] were extracted in 25 ml of aqueous 0.05 % sodium desoxycholate solution for 10–15 min and then centrifuged. All operations were carried out at 0–5° and the centrifugations performed at 15,000 × *g* for 10–15 min.

Graded quantities of solid $(NH_4)_2SO_4$ were added to the extract with constant stirring and the precipitated protein fractions collected by centrifugation. The precipitates were dissolved in isotonic saline and their conversion abilities determined using a heated isotonic saline extract of an equivalent amount of mitochondria as the source of cofactors. Protein was determined from separate aliquots using the Folin-Ciocalteu Reagent[11].

Conversion of PGA to CF

The optimal conditions reported for the conversion with rat liver[2,12] were maintained. The reactions were carried out in conventional Warburg flasks. Each vessel contained 200 μg PGA, 10 mg serine, and 3 ml of 20 % liver homogenate or the corresponding cellular fraction dissolved in 0.066 M phosphate buffer pH 6.4, final volume 5 ml. The reaction was allowed to proceed under N_2 at 37° for 100 min after which it was arrested by steaming the stoppered flasks for 5 min. CF was assayed using *Leuconostoc citrovorum* (American Type Cultures Collection No. 8081) as the test organism in a basal medium of SAUBERLICH AND BAUMANN[13]. Conversion values, after correction for endogenous CF, are expressed as μg CF formed by 1 g liver or the cellular fractions corresponding to 1 g liver.

DHPGA synthesis: This was studied by incubating PGA (200 μg) under N_2 for 1 h at 37° in 0.06 M phosphate buffer, pH 6.4, with isotonic saline liver supernatant (3.0 mg nitrogen) in a total volume of 3.0 ml. The product was then acidified, aerated and read at 420 mμ on a Beckman DU spectrophotometer against an enzyme blank in a reference cuvette[5]. For the zero blank, the reactants were mixed just before acidification.

Succinoxidase: Determinations of succinoxidase were carried out according to the method of SCHNEIDER AND POTTER[14].

Radioactivities: These were determined in infinitely thin measured portions evaporated to dryness on 25 mm diameter pyrex glass planchettes, using a helium-filled SL-16 flow gas counter in conjunction with a SC-51 Autoscaler (Tracer Lab. Inc., Waltham, Mass., U.S.A.) operating at 40–45 % efficiency.

RESULTS

Experiments using fractions from liver homogenates in isotonic saline

The conversion of PGA to CF did not proceed satisfactorily when isotonic sucrose was used as the medium for homogenation. This effect was not further investigated. However, conversion values for homogenates in isotonic saline were comparable to those obtained when aqueous phosphate buffer[12] was used for homogenation. The CF synthesized from added PGA was in a free and assayable form since chemical and enzymic liberation procedures only served to increase the endogenous levels (unpublished data).

No single cellular fraction of the isotonic saline homogenate was found to effect CF synthesis. A combination of the supernatant ($> 5000 \times g$) and the fraction 700 × *g* accounted for the entire conversion of the whole liver homogenate (Table I). By heat denaturation of either fraction or by substitution of the co-factors[3] (ATP, TPN and Mg^{++}) for the supernatant, CF synthesis did not proceed, indicating an enzymic contribution by each fraction.

The conversion was then shown to proceed in two subsequent stages, each separately terminable by heating the reaction system. The first stage is brought about by the supernatant enzymes, while the fraction 700 × *g* undergoes conversion in the presence of a formyl donor (Table I). The conversion did not proceed when this sequence was reversed. Aminopterin addition inhibited CF synthesis even when

TABLE I

CONVERSION OF PGA TO CF BY ISOTONIC SALINE HOMOGENATE FRACTIONS OF RAT LIVER

Conversion values were obtained as described in the METHODS section. Results are expressed as mean ± S.E.M.

Expt. No.	*Enzyme source*	*CF formed (μg/g liver)*
1	Total homogenate	1.52 ± 0.15
2	Fraction 700 × *g* and supernatant	1.50 ± 0.16
3	Fraction 5000 × *g* and supernatant	0.08 ± 0.02
4	Supernatant (1st step), then fraction 700 × *g* (2nd step), stepwise*	1.35 ± 0.18
5	Fraction 700 × *g*, then supernatant, stepwise*	0.16 ± 0.04
6	1st step aerobic, 2nd step anaerobic*	1.28 ± 0.25
7	1st step anaerobic, 2nd step aerobic*	0.37 ± 0.21
8	Aminopterin added in 2nd step*	0.04 ± 0.02

* The reaction was carried out stepwise. In the 1st step PGA was reacted with a cellular fraction for 50 min and the reaction stopped by steaming. The 2nd cellular fraction was then allowed to react for a further 50 min, before termination of the experiment. Unless otherwise specified, all incubations were anaerobic. Attention is drawn to Expt. No. 4 where serine addition was found necessary in the 2nd step. In Expt. Nos. 6 and 7 the steps are as in Expt. No. 4. Aminopterin, where added, amounted to 10 μg level per flask.

the substance was added in the latter stage where anaerobic conditions were most crucial for optimal conversion (Table I).

An observed increase in the optical density at 420 mμ, following acidification and exposure to air of the products of the supernatant reaction (Table II) was interpreted as indicating the formation of DHPGA, which is known to give a yellow degradation product[5, 15].

The cellular fraction sedimenting at 700 × g from isotonic saline homogenates accounted for 90 % of the total liver succinoxidase activity (Table III). When mitochondria labelled *in vivo*, by sedimentation from isotonic sucrose liver homogenates of rat killed 2 h after intraperitoneal injection of an aqueous solution of 1 mg [3-^{14}C]-serine (2.5 × 10^5 counts/min per 100 g body wt.), were added to a liver homogenate in isotonic saline and re-sedimented, it was observed (Table IV) that 70 % of the mitochondrial radioactivity was recoverable in fraction 700 × *g*. Fraction 5000 × *g* accounted for only 5 % of the activity, while 20 % was recovered in the supernatant (> 5000 × *g*).

Experiments using fractions from liver homogenates in isotonic sucrose

CF synthesis was demonstrable with purified nuclei and mitochondria prepared from isotonic sucrose homogenates and suspended in isotonic saline (Table III). The mitochondria were responsible for most of the conversion ability of the particulate fraction. The small conversion by the nuclear fraction was attributed to contaminating

TABLE II

CONVERSION OF PGA TO CF BY SUPERNATANT OF RAT LIVER ISOTONIC SALINE HOMOGENATES

See METHODS section for details.

No.	*Optical density at 420 mμ*	
	0 h	*1 h*
1	0.12	0.41
2	0.08	0.33
3	0.09	0.47

TABLE III

COMPARATIVE STUDIES WITH CELLULAR FRACTIONS FROM ISOTONIC SALINE AND ISOTONIC SUCROSE HOMOGENATES OF RAT LIVER

For details of procedure see METHODS section. Results are expressed as mean ± S.E.M.

Medium of homogenisation (isotonic)	*Source of enzyme*	*CF formed (μg/g liver)*	*Succinoxidase activity (μl O_2/h/g liver)*
Saline	Whole homogenate	1.52 ± 0.15	3868 ± 122
Sucrose	Whole homogenate	0.68 ± 0.14	3917 ± 145
Saline	Fraction 700 × *g* and supernatant	1.50 ± 0.16	3595 ± 155
Sucrose	Nuclei*	0.28 ± 0.03	495 ± 36
Sucrose	Mitochondria*	1.19 ± 0.12	2908 ± 55
Sucrose	Nuclei and mitochondria*	1.49 ± 0.18	3508 ± 88

* Prepared from isotonic sucrose homogenates and suspended in isotonic saline.

mitochondria, since this fraction was not completely devoid of succinoxidase activity (Table III).

The protein fraction, separating at between 30–50% $(NH_4)_2SO_4$ saturation of the 0.05% aqueous sodium desoxycholate mitochondrial extract, accounted for 90% of activity of the mitochondria (Table V). Fractions separating at between

TABLE IV

DISTRIBUTION OF MITOCHONDRIA IN CELLULAR FRACTIONS FROM ISOTONIC SALINE LIVER HOMOGENATES

Labelled mitochondria were obtained by fractionation of isotonic sucrose liver homogenates of rats killed 2 h after intraperitoneal injection of an aqueous solution containing 1 mg [3-^{14}C]serine (2.5×10^5 counts/min/100 g body weight). These were added to a liver homogenate in isotonic saline, re-sedimented and washed at 700 × *g* and 5000 × *g* for 10 min, each. Radioactivities of the particulate and supernatant (> 5000 × *g*) fractions were determined as described in the METHODS section. Results are expressed as mean ± S.E.M.

Fraction	*Activity (counts/min)*	*% of total activity*
Total homogenate	420 ± 32	100
Fraction 700 × *g*	295 ± 20	70
Fraction 5000 × *g*	30 ± 4	7
Supernatant (> 5000 × *g*)	88 ± 6	21

TABLE V

PARTIAL PURIFICATION OF THE CF-FORMING ENZYMES OF RAT LIVER MITOCHONDRIA

For details of assay see the METHODS section. Specific activities are expressed as mμg CF formed/mg protein.

Enzyme preparation	*CF formed (μg/g liver)*	*Apparent yield (%)*	*Protein content (mg/g liver)*	*Specific activity*
Homogenate	1.52	100	220.0	6.7
Mitochondria	1.21	79	44.2	27.5
0–60% $(NH_4)_2SO_4$ fraction*	0.40	26	28.4	14.5
30–50% $(NH_4)_2SO_4$ fraction*	0.38	25	7.9	48.9
30–50% $(NH_4)_2SO_4$ fraction with cofactors**	1.32	88	7.9	167.1

* PGA to CF conversion abilities were studied with protein fractions precipitating at different $(NH_4)_2SO_4$ saturations of the 0.05% desoxycholate extract of mitochondria, in the presence of a heated isotonic saline extract of an equivalent amount of mitochondria to provide the cofactors.

** In this case the source of cofactors was a mixture containing 5 mg $MgCl_2$, 1 mg TPNH, 1 mg ATP, 3 mg homocysteine and 1 mg pyridoxal phosphate, in 5 ml of the reaction system.

TABLE VI

FORMYL DONORS AND THE CONVERSION OF PGA TO CF BY RAT LIVER MITOCHONDRIA

Formyl donors were added at 0.1 mmole level per flask and the conversion values obtained as described in the METHODS section. Results are expressed as mean ± S.E.M.

Formyl donor	*CF formed (μg/g liver)*
Serine	1.19 ± 0.12
Formate	0.52 ± 0.07
Formaldehyde	0.07 ± 0.01

0–30 % and 50–60 % $(NH_4)_2SO_4$ saturation showed negligible activities on analysis.

Formyl donors: In the next experiment the effect of different formyl donors on the CF synthesis by the mitochondria was studied (Table VI). Serine, formate and formaldehyde are effective, in that order, as formyl donors.

Cofactors and other requirements: The heated extract of mitochondria which supplied cofactors for the conversion effected by the partially purified enzyme precipitate (Table V) could be completely replaced by a mixture containing TPNH, ATP, $MgCl_2$, homocysteine and pyridoxal phosphate, with vastly improved yields (Table VII). The lower yields with the former were probably due to the absence of a TPNH regenerating system in the heated mitochondrial extract (unpublished data). By optional omission of each component of the mixture, it was found (Table VII) that the TPNH and ATP additions were most crucial for optimal conversion, followed by Mg^{++}, homocysteine and pyridoxal phosphate.

Reducing conditions: Table VIII would indicate that reducing conditions are essential for conversion and act non-specifically. A nitrogen atmosphere favoured conversion better than ascorbic acid addition. Further, in a N_2 atmosphere, ascorbic acid addition was without effect.

TABLE VII

EFFECT OF VARIOUS ADDITIONS ON THE CONVERSION OF PGA TO CF

The partially purified enzyme precipitate obtained at 30–50 % $(NH_4)_2SO_4$ saturation of the 0.05 % deoxycholate extract of mitochondria was used as the enzyme source. The additions were as described in Table V and the conversion values, representing the average of 4 different experiments, were determined omitting each of the components in turn.

Omission	*CF formed (μg/g liver)*
Nil	1.32
TPNH*	0.13
ATP	0.40
$MgCl_2$	0.51
Homocysteine	0.84
Pyridoxal phosphate	1.13

* TPN showed an effect only in the presence of isocitrate and isocitric dehydrogenase (supernatant).

TABLE VIII

REDUCING CONDITIONS FOR THE CONVERSION OF PGA TO CF BY RAT LIVER MITOCHONDRIA

Freshly neutralised ascorbic acid was added in 10 mg amounts per flask. Conversion values were obtained as described in the METHODS section. Results are expressed as mean ± S.E.M.

Ascorbic acid addition	*Under N_2*	*CF formed (μg/g liver)*
+	—	0.66 ± 0.04
—	+	1.19 ± 0.12
+	+	1.10 ± 0.12
—	—	0.02 ± 0.01

DISCUSSION

Experiments with cellular fractions from liver homogenates in isotonic saline indicated that the conversion proceeded in two subsequent stages, the first brought about by the supernatant ($> 5000 \times g$) enzymes and the second by the particulate enzymes of fraction $700 \times g$.

Reduction of PGA to THPGA precedes formylation and aminopterin addition inhibits conversion by blocking the reduction of PGA[16, 17]. In a bacterial system WRIGHT AND ANDERSON[5] indicated that aminopterin did not inhibit the initial reduction of PGA to DHPGA. In our studies aminopterin inhibited CF synthesis when added in the final stage (fraction $700 \times g$) reaction where the reducing conditions were most crucial and the addition of a formyl donor was found necessary (Table I). This would suggest that reduction of DHPGA to THPGA and the subsequent formylation were mediated by enzymes of the fraction $700 \times g$. Reduction of PGA to DHPGA by the supernatant enzymes is implied in Table II. To confirm this, CF synthesis from synthesized DHPGA[18] corresponding to 1.50 µg/g liver (average of 4 different incubations) was demonstrable under optimal conditions in the presence of fraction $700 \times g$ together with the heated supernatant and serine.

Succinoxidase determinations (Table III) indicated the presence of mitochondria in the particulate fraction sedimenting at $700 \times g$. Experiments with purified cellular fractions showed that the mitochondrion was the active site of conversion (Table III). Further labelled work (Table IV) suggested agglutination and disintegration of the mitochondria in isotonic saline, followed by release of intra-mitochondrial contents. HOGEBOOM, SCHNEIDER AND PALLADE[19] have reported similar observations on mitochondria prepared in isotonic saline. Thus it would seem that the enzyme systems in the mitochondria could be divided into the one which brings about the initial reduction of PGA to DHPGA and the other which effect the final reduction to THPGA followed by formylation to CF active derivatives; the former was easily released into the supernatant in isotonic saline, while the latter was more firmly bound in the mitochondrion.

The distribution of CF-synthesising enzymes in rat liver differs from that in chicken liver, where they are apparently more readily soluble and reside in the supernatant[17]. Extraction of the mitochondria by 0.05 % aqueous sodium desoxycholate solubilized the enzyme complex and 90 % of the activity was recovered in the protein fraction precipitating at between 30 and 50 % $(NH_4)_2SO_4$ saturation (Table V). These results are in accord with those of DEODHAR, SAKAMI AND STEVENS[20] who have reported a solubilized preparation from rat liver particles that accumulated a CF-active product when THPGA was activated in the glycine–serine interconversion. NADKARNI AND SREENIVASAN[21] attributed 70 % of the liver activity for the reverse reaction involving serine synthesis with CF utilisation to the mitochondria.

A need for pyridoxal phosphate (Table VII) in the overall serine hydroxymethylase reaction has constantly been observed[22]. DOCTOR *et al.*[3] have also reported stimulatory effects of Mg^{++} and homocysteine. The latter probably enhance the utilisation of 1-C fragments in CF synthesis by forming a homocysteine-C_1 common precursor in 1-C transfers[23].

The multiplicity of the formyl donors, essential for the conversion, lends uncertainty to the exact nature of the active intermediates formed. In the present study serine was the most effective (Table VI). For rat liver enzymes, Elwyn *et al.*[24] have also reported serine to be the most effective precursor of the methyl group of choline, a process in which the C:H bond of the β-C of serine remained unaltered. This would suggest that the N^{10}-hydroxymethyl derivative of THPGA[25] was formed in an ATP dependent reaction[26] without the obligatory formation of the formyl derivative. The two derivatives are known to exist in a redox equilibrium with a TPN-linked dehydrogenase which has been isolated in a purified form[27]. This reaction could be compared with the known TPNH-linked reduction of PGA to DHPGA[5,6] and THPGA[18,28]. Under anaerobic conditions labile THPGA and its N^{10}-formylated derivatives undergo non-enzymic degradation to CF, the stable N^{5}-formyl derivative[9]. As observed by Donaldson and Keresztesy[29], reducing agents act only as non-specific stabilizers (Table VIII). Under a nitrogen atmosphere the addition of ascorbic acid had no effect (Table VIII). The latter is known to inhibit specifically the aerobic cleavage of CF[30].

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REFERENCES

[1] H. P. Broquist and A. R. Kobler, *J. Biol. Chem.*, 202 (1953) 59.
[2] C. A. Nichol and A. D. Welch, *Proc. Soc. Exptl. Biol.*, 74 (1950) 52.
[3] V. M. Doctor, J. R. Couch and J. B. Trunnell, *Proc. Soc. Exptl. Biol.*, 87 (1954) 228.
[4] C. A. Nichol, *J. Biol. Chem.*, 204 (1953) 469.
[5] B. E. Wright and M. L. Anderson, *J. Am. Chem. Soc.*, 79 (1957) 2027.
[6] J. M. Peters and D. M. Greenberg, *Biochim. Biophys. Acta*, 32 (1959) 273.
[7] R. L. Blakley, *Biochem. J.*, 58 (1954) 448.
[8] G. R. Greenberg, *Federation Proc.*, 13 (1954) 745.
[9] C. A. Nichol, A. H. Anton and S. F. Zakrzewski, *Science*, 121 (1955) 275.
[10] G. H. Hogeboom and W. C. Schneider, *J. Biol. Chem.*, 183 (1950) 123.
[11] E. Layne, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. III, Academic Press, Inc., New York, 1957, p. 447.
[12] C. A. Nichol, *Federation Proc.*, 11 (1952) 452.
[13] H. E. Sauberlich and C. A. Baumann, *J. Biol. Chem.*, 176 (1948) 165.
[14] W. C. Schneider and V. R. Potter, *J. Biol. Chem.*, 149 (1943) 217.
[15] R. L. Blakley, *Biochem. J.*, 65 (1957) 331.
[16] R. L. Blakley, *Nature*, 173 (1954) 729.
[17] V. M. Doctor, *J. Biol. Chem.*, 222 (1956) 959.
[18] S. Futterman, *J. Biol. Chem.*, 228 (1957) 1031.
[19] G. H. Hogeboom, W. C. Schneider and G. E. Pallade, *J. Biol. Chem.*, 172 (1948) 619.
[20] S. D. Deodhar, W. Sakami and A. Z. Stevens, *Federation Proc.*, 14 (1955) 201.
[21] G. B. Nadkarni and A. Sreenivasan, *Proc. Indian Acad. Sci.*, 47B (1958) 189.
[22] F. M. Huennekens, M. J. Osborn and H. R. Whiteley, *Science*, 128 (1958) 120.
[23] P. Berg, *J. Biol. Chem.*, 205 (1953) 145.
[24] D. Elwyn, A. Weissbach, S. S. Henry and D. B. Sprinson, *J. Biol. Chem.*, 213 (1955) 281.
[25] L. Jaenicke, *Biochim. Biophys. Acta*, 17 (1955) 588.
[26] J. M. Peters and D. M. Greenberg, *J. Biol. Chem.*, 226 (1957) 329.
[27] Y. Hatefi, M. J. Osborn, L. D. Kay and F. M. Huennekens, *J. Biol. Chem.*, 227 (1957) 637
[28] S. F. Zakrzewski and C. A. Nichol, *Biochim. Biophys. Acta*, 27 (1958) 425.
[29] K. O. Donaldson and J. C. Keresztesy, *J. Biol. Chem.*, 234 (1959) 325.
[30] J. S. Dinning, J. T. Sime, P. S. Work, B. Allen and P. L. Day, *Arch. Biochem. Biophys.*, 66 (1957) 114.

STUDIES ON THE ENZYMIC DEHYDROGENATION OF DEUTERATED SUCCINATE

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SUMMARY

1. Increasing the deuterium content of succinic acid decreased the rate of dehydrogenation by succinoxidase. In cell-free systems, completely deuterated succinate was dehydrogenated at about one-sixth the rate of ordinary succinate.

2. Solvent effects of D_2O were of less importance. In pure D_2O, the dehydrogenation of ordinary succinate was inhibited about 35 %; in 30 % D_2O, the inhibition was only 5 %.

3. Metabolism of succinate by liver slices showed the same effect of deuteration of the substrate that was observed with cell-free preparations, except that the isotope effect was somewhat less.

INTRODUCTION

In the study of the effects of heavy water on animals, homogenates of liver and kidney were assayed for a number of enzymes[1]. Only liver catalase and DPN–cytochrome *c* reductase were markedly decreased (65 % of control activity in moribund animals). Succinic dehydrogenase was diminished slightly in both liver and kidney.

However, these studies were carried out with substrates containing ordinary hydrogen. It was thus of interest to study the effects of substitution of partially and completely deuterated substrates in the assay systems. THORN[2] had previously shown that dideutero- and tetradeuterosuccinate (actually containing 42 and 77 atom % D in the methylene groups) were dehydrogenated significantly more slowly than ordinary succinate. We have extended THORN's observations, using both more and less completely deuterated succinates, and have also compared the dehydrogenation of these succinates in both H_2O and D_2O.

METHODS

Tetradeuterosuccinate was prepared by catalytic hydrogenation of acetylene dicarboxylic acid by deuterium gas. The details of the synthesis will be published elsewhere[3]. The deuterium content of tetradeuterosuccinic acid was 96.2 atom %; the minimum value for the methylene groups was thus 94.6 atom % D, considerably higher than the 77 atom % reported by THORN[2].

Dideuterosuccinate was prepared by reduction of either fumaric or maleic acid by zinc in 5 N D_3PO_4. The acid was extracted with ether and recrystallized from D_2O. The deuterium content of several preparations ranged from 63.7 to 65.0 atom %, corresponding to a minimum of 46 to 48 % deuterium on the methylene carbons.

The reduction of fumaric acid by zinc in mixtures of D_3PO_4 and H_3PO_4 was used to prepare succinates containing smaller amounts of deuterium. From a mixture of phosphoric acids containing 60 atom % D, succinate with 15.6 atom % D in the methylene groups was obtained. It was necessary to use a mixture with 80 atom % D to prepare succinate with approximately 30 atom % D. There is thus an appreciable discrimination between H and D in this reaction.

Various sources of succinoxidase were employed: homogenized rat kidney, mitochondria prepared from rat liver[4], and a preparation from beef heart[5]. In one experiment, an attempt was made to obtain an enzyme preparation with an appreciable percentage of non-exchangeable deuterium: subtotal hepatectomy was performed on a rat with about 25 % of its body water replaced by D_2O; after operation, the rat was given 30 % D_2O to drink for two weeks; the rat was then killed, and mitochondria were prepared from the regenerated liver. Succinoxidase was usually assayed with cytochrome *c* as the acceptor[6]; methylene blue[5] and phenazine methasulfate[7] were used in some experiments. In most cases, the oxygen consumption occurring between 5 and 25 min after addition of the substrate was used as the basis of comparison.

For the study of oxidative phosphorylation with deuterosuccinates as the substrate, the system of LARDY AND WELLMAN was used[8].

Liver slices were cut with a Stadie-Riggs microtome. Oxygen was used as the gas phase, and the final concentration of succinate in Krebs–Ringer–phosphate was 0.03 M. Oxygen consumption was measured over a 40 min interval.

RESULTS

Oxidation of deuterosuccinates in D_2O

The oxidation of tetradeuterosuccinate was studied in a nearly completely deuterated system. The only hydrogen (*i.e.*, protium) present was in the acceptors (cytochrome *c*, methylene blue, or phenazine methosulfate) and in the enzyme preparation. For comparison, ordinary succinate in both D_2O- and H_2O-systems and tetradeuterosuccinate in H_2O were studied simultaneously. Two experiments were carried out, one with a preparation of mitochondria from a normal rat, the other with mitochondria prepared from regenerated liver of a rat drinking 30 % D_2O. In both experiments the mitochondria were washed and resuspended in 0.25 M sucrose in 100 D_2O. The results of these experiments are shown in Table I. No differences were observed when different acceptors were used; further, the mitochondrial preparation from the deuterated rat had the same Q_{O_2} succinate as did that from the normal animal. Hence all of the data have been pooled, so that each entry represents the average of six determinations, each of which was carried out in duplicate.

It is clear that in the case of tetradeuterosuccinate virtually the same degree of inhibition was observed in D_2O as in H_2O; the solvent effects of D_2O were almost completely masked by the primary isotope effect. The dehydrogenation of ordinary succinate was somewhat inhibited in D_2O, but the rate of dehydrogenation of this

TABLE I

RELATIVE RATES OF DEHYDROGENATION OF DEUTEROSUCCINATES IN H_2O AND D_2O

Atom % D		*No. determinations*	*Per cent control activity*			
			H-succinate		*D-succinate*	
Substrate	*Medium*		*In H_2O*	*In D_2O*	*In H_2O*	*In D_2O*
94.6	100	6	100	65.9	17.2	16.1
28.8	30	3	100	95.4	82.8	80.4
15.6	15	4	100	94.5	89.9	87.0

compound in D_2O was much faster than that of tetradeuterosuccinate in either H_2O or D_2O.

Table I also shows the results of similar experiments carried out with 30 and 15 % deuterosuccinates, in both H_2O and 30 or 15 % D_2O. The higher concentration was chosen because it represents the maximum amount of replacement of body water by D_2O which a rat can tolerate. The lower concentration produces very few effects in the living animal. In these experiments only cytochrome *c* was used as the acceptor; rat kidney homogenate was the source of the enzyme.

Both the 30 and 15 % deuterosuccinates were dehydrogenated more slowly than ordinary succinate in H_2O, the former about 20 %, and the latter about 10 %. D_2O in the concentrations employed had a very slight inhibitory action.

Dehydrogenation of deuterosuccinates in H_2O

In Fig. 1 are shown the relative rates of dehydrogenation of succinates containing 0, 15.6, 28.8, 47.5, and 94.6 atom % D. The data of THORN[2] are also shown for 44 and 72 atom % D. It is evident that there is a virtually linear relationship between activity and extent of replacement of hydrogen by deuterium.

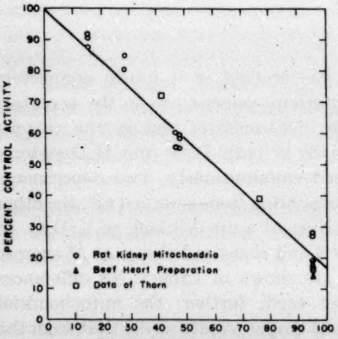

Fig. 1. Effect of deuteration of succinate on succinoxidase activity of rat kidney homogenate or beef heart preparation.

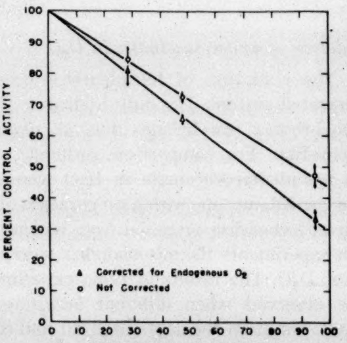

Fig. 2. Effect of deuteration of succinate on succinoxidase activity of slices of rat liver.

Dehydrogenation by liver slices

Fig. 2. shows the effect of deuteration of succinate on the rate of oxidation by slices of rat liver. As with homogenates, the response was linear. However, the effect was not quite so marked, even when the data were corrected for oxygen consumption in the absence of succinate. Tetradeuterosucinate was oxidized at 33 % of the control rate by liver slices, but only 22 % by kidney homogenates or beef heart preparations. The reason for this discrepancy is doubtless attributable to the fact that the succinoxidase system was not acting at maximum potential in the slices; under optimum conditions, liver homogenates oxidize succinate at 4 to 5 times the rate observed in slices.

Oxidative phosphorylation

Table II shows that, under the conditions of assay for this system, only tetradeuterosuccinate was dehydrogenated more slowly than ordinary succinate. The esterification of inorganic phosphate was similarly reduced, but the fact that the P/O ratios did not differ significantly provides evidence that the efficiency of the system was not impaired.

TABLE II

OXIDATIVE PHOSPHORYLATION WITH DEUTEROSUCCINATES AS SUBSTRATES

Atom % D Succinate	*μMP/ mg N*	*μ atoms O/ mg N*	*P/O ratio*
0	26.0	13.9	1.9
28.8	24.0	14.2	1.7
47.9	26.5	15.0	1.8
94.6	18.1	9.5	1.9

DISCUSSION

On the basis of measurements of the Michaelis constants, THORN estimated that the affinity between succinoxidase and ordinary succinate was 1.45 times that between the enzyme and succinate containing 77 atom % D (see ref. 2). It is reasonable to believe that the affinities of the other deuterosuccinates would differ from that of ordinary succinate, probably in proportion to the deuterium content.

In these experiments, dideutero- and tetradeuterosuccinate may be considered as homogeneous compounds, for which meaningful Michaelis constants could be derived. However, the 15 and 30 % deuterosuccinates represent mixtures of succinic acid containing, in unknown proportions, 0, 1, and 2 atoms of deuterium. One would thus expect that the molecules containing no deuterium would be preferentially dehydrogenated. This is probably the reason why the dehydrogenation of 15 and 30 % deuterosuccinate was slightly faster than would be anticipated from the observations with dideutero- and tetradeuterosuccinates (Fig. 1).

The data presented in Table I show that the solvent effects of D_2O are much less important than the primary isotope effects in determining the rate at which succinate is oxidized. The rate of dehydrogenation of ordinary succinate in 100 % D_2O was reduced to about the same level (65 %) as was observed with dideuterosuccinate in H_2O. Levels of D_2O below 30 % had very little effect on the dehydrogenation of succinate, normal or deuterated. Some years ago THUNBERG[7] observed that the "dehydrogenase" activity of an extract of peas was inhibited by D_2O; the effect was linear, and in pure D_2O the inhibition was 30 %, not much different from the 35 % noted for succinoxidase in these experiments. JACOBSOHN AND SOARES, on the other hand, reported that the malic dehydrogenase activity of a preparation of cucumber seeds was enhanced in pure D_2O (see ref. 10).

Since the rate of oxidation of tetradeuterosuccinate was uniformly slow in relation to that of ordinary succinate regardless of the acceptor—phenazine methosulfate, methylene blue, or cytochrome *c*—it is probable that the initial transfer of hydrogen is the rate-limiting step. If it were not, one would expect that a higher rate would be observed with phenazine or with methylene blue (or both) than with cytochrome *c*, since the three acceptors function at different levels of the electron transport mechanism.

The lack of effect of all but the completely deuterated succinate in the oxidative phosphorylation system indicates that the rate of oxygen consumption was determined by factors other than the rate of dehydrogenation of the substrate. Only when the deuterium content of succinate was increased to some value above 50 % of the normal did this reaction become the rate-limiting step. The presence of tetradeuterosuccinate did not affect the efficiency of phosphate esterification; in this system, the solvent effects of D_2O may be of greater importance, since 60 % D_2O decreased the P/O ratio by about 10 % (see ref. 11).

The influence of deuterated substrates on the development of the syndrome of heavy water intoxication in mammals is not easy to assess. All dehydrogenation reactions would probably be slowed somewhat, probably to about the same extent as succinate dehydrogenation, since the rate-limiting step seems to be the initial hydrogen transfer. Thus on the basis of the experiments with both liver slices and cell-free preparations, one would predict that in an animal with 30 % of his body water replaced by D_2O there would be a decrease in dehydrogenase activity to about 80 % of normal. Whether such a decrease actually takes place *in vivo* is open to question. BARBOUR reported that the metabolic rate of D_2O-treated mice was increased in the early stages of D_2O intoxication (10 to 20 % replacement) but fell to very low levels shortly before death[12]. Because of the profound adaptive changes which are known to occur in animals drinking D_2O (see ref. 1), these data are difficult to interpret. Possibly a study of the effects of D_2O on the metabolic rate of hypophysectomized animals would be useful.

One factor complicating the extension of results of *in vitro* experiments to the intact animal is the enzymic discrimination between deuterated and non-deuterated substrates[2]. Another is an enzyme-catalyzed exchange between the deuterium of the substrate (ordinarily non-exchangeable) and the hydrogen of the medium. The latter has been described by several authors; the magnitude of this exchange is uncertain, however. WEINMANN *et al.*[13] considered it to be large, while ENGLARD AND COLOWICK[14] found it to be very small in relation to succinate oxidation unless fumarate was present. In the short-term *in vitro* experiments reported here, the enzyme-catalyzed exchange was probably negligible; it might be appreciable in the intact animal. The effect of enzymic discrimination would be the accumulation of the more heavily deuterated succinates; however, the exchange reaction could probably re-establish the same equilibrium between substrate and medium.

ACKNOWLEDGEMENTS

Tetradeuterosuccinate was synthesized by T. R. SATO. Analyses for deuterium content were carried out by Dr. H. L. CRESPI. Triply distilled D_2O was made available through Dr. J. J. KATZ, whom we thank for his interest in this work.

REFERENCES

[1] J. J. KATZ, H. L. CRESPI, R. J. HASTERLIK, J. F. THOMSON AND A. J. FINKEL, *J. Natl. Cancer Inst.*, 18 (1957) 641.
[2] M. B. THORN, *Biochem. J.*, 49 (1951) 602.
[3] T. R. SATO AND J. H. POMEROY, unpublished observations.
[4] J. F. THOMSON AND F. J. KLIPFEL, *Arch. Biochem. Biophys.*, 70 (1957) 224.
[5] E. C. SLATER, *Biochem. J.*, 45 (1949) 8.
[6] W. C. SCHNEIDER AND V. R. POTTER, *J. Biol. Chem.*, 149 (1943) 217.
[7] E. B. KEARNEY AND T. P. SINGER, *J. Biol. Chem.*, 219 (1956) 963.
[8] H. A. LARDY AND H. WELLMAN, *J. Biol. Chem.*, 195 (1952) 215.
[9] T. THUNBERG, *Enzymologia*, 3 (1937) 56.
[10] K. P. JACOBSOHN AND M. SOARES, *Compt. rend. soc. biol.*, 126 (1937) 592.
[11] J. F. THOMSON AND F. J. KLIPFEL, *Am. J. Physiol.*, 197 (1959) 1128.
[12] H. G. BARBOUR, *Yale J. Biol. Med.*, 9 (1937) 651.
[13] E. O. WEINMANN, M. G. MOREHOUSE AND R. J. WINZLER, *J. Biol. Chem.*, 168 (1947) 717.
[14] S. ENGLARD AND S. F. COLOWICK, *J. Biol. Chem.*, 221 (1956) 1019.

OXYDATION DU SULFITE EN SULFATE PAR LA RACINE D'AVOINE

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SUMMARY

Oxidation of sulphite to sulphate by wheat roots

Wheat roots contain an enzymic system catalysing the oxidation of sulphite to sulphate. The optimum pH of the non-purified system is 7.0 in a phosphate buffer. The metal-chelating agents used are shown to inhibit the oxidation.

INTRODUCTION

Au cours de l'étude de la réduction du sulfate en sulfite dans la feuille de tabac[1], il est apparu que le sulfite est simultanément réoxidé en sulfate. La rapidité de cette oxydation est telle qu'elle compromet une mesure exacte de la quantité de sulfite formé par réduction. Pour tenter d'éliminer cette difficulté nous avons entrepris l'examen des modalités d'oxydation du sulfite par les tissus végétaux. Dans des essais préliminaires, nous avons utilisé avec des résultats du même ordre les feuilles de tabac, de physalis, d'avoine, et les racines des mêmes plantes. Nous avons finalement retenu la racine d'avoine comme matériel de départ pour des raisons de commodité: développement de ces racines sans support solide, en toutes saisons, et absence des pigments foliaires.

L'oxydation du sulfite en sulfate dans les végétaux a déjà fait l'objet des travaux de Tager et Rautanen[2,3]. Ces auteurs utilisent les mitochondries isolées des feuilles d'avoine. Sans conclure sur le mécanisme de l'oxydation étudiée, Tager et Rautanen indiquent cependant qu'il est différent de celui décrit par Handler[4,5] pour l'oxydation du sulfite par les tissus animaux.

Le présent travail donne quelques aspects de l'oxydation du sulfite en sulfate en présence d'un extrait aqueux de racine d'avoine, et montre que cette réaction est catalysée par un agent thermolabile, non dialysable et qui possède un métal. Ce catalyseur est très actif puisque l'on peut observer, au pH optimum de l'oxydation à 37°, et en présence d'une concentration en sulfite $5 \cdot 10^{-3}\,M$, un Q_{O_2} rapporté à 1 mg d'azote total supérieur à 24,000.

Mise en évidence et influence du pH sur l'oxydation du sulfite en sulfate

Partie expérimentale: Les racines sont prélevées de plantules d'avoine germée développées à l'obscurité. Les graines et les plantules sont lavées chaque jour à l'eau ordinaire et rincées à l'eau distillée. Elles se développent sur un tamis en matière plastique dans une enceinte saturée de vapeur d'eau. La récolte des racines a lieu 5 jours après la germination. Les racines sont lavées à l'eau distillée et broyées à 0° au mortier dans un tampon phosphate $0.1\,M$ pH 7.0 contenant du chlorure de sodium à la concentration de $0.35\,M$. On utilise 1 ml de tampon/g de racines fraîches. Le broyat est filtré sur mousseline et est centrifugé à 0° à $1{,}600 \times g$ pendant 10 min.

L'oxydation du sulfite est mesurée par voie manométrique avec l'appareil de Warburg, à la température de 37° et avec une agitation correspondant à 220 alternances/min. On n'a pas utilisé de potasse dans la cupule centrale des cellules de Warburg, des essais préliminaires en ayant montré l'inutilité. Chaque essai comporte, sauf mention contraire: sulfite de sodium 9.5 µmoles, soit 0.2 ml de sa solution aqueuse, broyat 0.1 ml, tampon phosphate ou Tris $0.05\,M$ pH 7.0. Le volume total est de 2 ml. On attend 10 min pour obtenir l'équilibre de la température avant de mettre le sulfite en précence du broyat. Toutes les solutions aqueuses sont faites dans de l'eau d'abord purifiée par échangeurs d'ions, puis distillée dans un appareil en pyrex.

L'estimation du sulfite introduit ou restant à la fin d'une expérience est faite par iodométrie. La variation du pH du milieu est obtenue par l'emploi de tampons phosphate ou Tris $0.05\,M$. La mesure du pH n'est faite que lorsque tous les réactifs utilisés ont été introduits.

Résultats: L'oxydation du sulfite par le broyat de racines est totale. 9.5 µmoles de sulfite correspondent à une consommation d'oxygène théorique de 105 µl. La consommation d'oxygène au cours de nos essais se stabilise vers 80–95 µl. La différence correspond à une auto-oxydation du sulfite pendant les 10 min d'attente, le manomètre étant ouvert, nécessaires à l'équilibre de la température. Ce point a été vérifié par la mesure du sulfite restant après ces 10 min, et par le fait qu'il n'y a plus de sulfite décelable par iodométrie lorsque la consommation d'oxygène a atteint son maximum.

La vitesse d'oxydation est fonction de la quantité de broyat présente (Fig. 1). Le broyat bouilli ne conduit qu'à une lente absorption d'oxygène. L'influence du pH sur la vitesse d'oxydation du sulfite en présence de broyat de racines d'avoine est données par la Fig. 2. Pour le calcul du Q_{O_2} on a utilisé la consommation d'oxygène pendant les six premières minutes de la réaction. On voit que le pH optimum se situe au voisinage de pH 7. Le Q_{O_2} pour un tel broyat non purifié est supérieur à 24,000, ce qui témoigne de l'intensité de la réaction étudiée. Les résultats sont semblables que l'on se trouve en présence d'un tampon phosphate ou d'un tampon Tris.

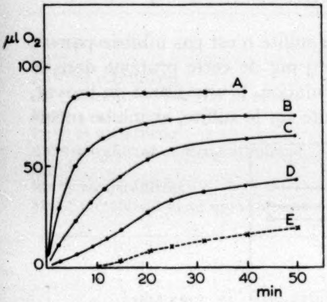

Fig. 1. Influence de la quantité de broyat de racines sur la vitesse d'oxydation du sulfite. Sulfite, 9.5 μmoles; broyat, 600 μg d'azote total/ml; tampon phosphate, 0.05 *M*; pH, 7.2, volume total, 2 ml; température, 37°. A, 0.3 ml de broyat; B, 0.1 ml; C, 0.04 ml; D, 0.02 ml; E, 02. ml de broyat bouilli.

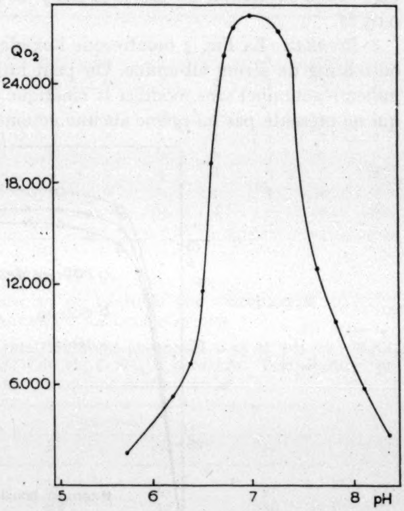

Fig. 2. Influence du pH sur l'oxydation du sulfite par le broyat de racines d'avoine. Sulfite, 9.5 μmoles; broyat, 0:2 ml (30 μg N total); tampon phosphate, 0.05 *M*; volume total, 2 ml; température, 37°. Le Q_{O_2} est calculé à partir de la quantité d'oxygène absorbée pendant les 6 premières minutes, déduction faite de l'oxygène utilisé par un témoin comportant du broyat chauffé.

Nature possible du catalyseur de l'oxydation du sulfite en sulfate présent dans les racines d'avoine

Que le chauffage jusqu'à ébullition du broyat annule pendant les 10 premières minutes l'oxydation du sulfite et ne permette après qu'une lente consommation d'oxygène n'est pas une preuve contraignante à elle seule, de l'existence d'un système enzymatique oxydant cet ion. En effet, les nombreux travaux consacrés[6] à l'autooxydation du sulfite soulignent le rôle catalytique extrèmement accentué des métaux et en particulier du cuivre[7]. On peut alors penser que la dénaturation des protéines du broyat par la chaleur fait apparaître des groupes qui complexent les traces métalliques présentes, et par suite, provoque une inhibition de la catalyse inorganique.

Pour montrer que la catalyse inorganique ne peut rendre compte de l'oxydation du sulfite en présence de broyat de racines, nous avons fait les expériences suivantes:

Partie expérimentale: Sans modifier le protocole décrit dans la première section, on procède premièrement à l'addition au milieu d'une solution de sérum albumine (0.3, 0.6, 3 mg), ou d'ultrafiltrat de broyat, obtenu par passage forcé de celui-ci à travers une membrane de cellophane; deuxièmement à la comparaison de l'oxydation du sulfite obtenue en présence de Cu^{++}, Fe^{++} ou Mn^{++} introduits sous forme de sulfate et de concentration finale $1 \cdot 10^{-5}$ *M*, et de celle à laquelle conduit le broyat de racines. Ce parallèle a été examiné à divers pH en présence d'un tampon Tris 0.05 *M*, de sérum albumine 1 mg et d'ultrafiltrat 0.4 ml; troisièmement à l'étude de l'influence du Co^{++}, Mo sous forme de molybdate d'ammonium, Mg^{++}, Zn^{++} et Cd^{++} sur l'oxydation du sulfite à pH 7 en présence de tampon phosphate ou Tris 0.05 *M*.

Résultats: La Fig. 3 montre que l'oxydation du sulfite n'est pas inhibée par 0.3 ou 0.6 mg de sérum albumine. On peut introduire 3 mg de cette protéine dans le milieu réactionnel sans modifier la cinétique de l'oxydation. L'ultrafiltrat du broyat, qui ne présente par lui-même aucune action oxydante sur le sulfite, et inhibe même complètement son oxydation spontanée, ne modifie pas l'oxydation du sulfite lorsqu'il est ajouté au broyat de racines. Sur la base de ces données il nous parait difficile d'admettre que le catalyseur de l'oxydation du sulfite présent dans le broyat de racines puisse être un ion métallique ou un complexe métallique de faible poids moléculaire. Ce point de vue est confirmé par l'examen de la catalyse de l'oxydation du sulfite que les ions métalliques introduits provoquent. Les Tableaux I et II montrent que seul l'ion cuivre dans les conditions utilisées, peut catalyser l'oxydation du sulfite. Toutefois, l'ultrafiltrat ou l'ultrafiltrat et la sérum albumine inhibent cette oxydation.

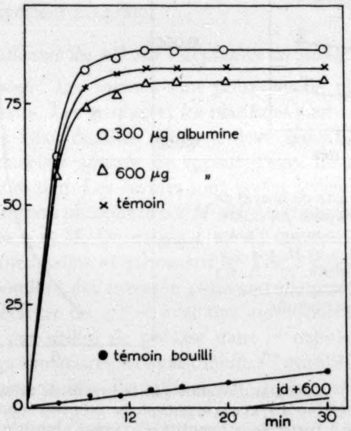

Fig. 3. Oxydation du sulfite par le broyat de racines en présence de sérum albumine. Sulfite, 9.5 µmoles; broyat, 0.1 ml (78 µg N total); tampon phosphate, 0.05 *M*; pH 7.0, sérum albumine, 300 et 600 µg; volume total, 2 ml; température, 37°.

Le molybdène sous forme de molybdate d'ammonium, l'ion cobalt à la concentration de $1 \cdot 10^{-3}\ M$ sont sans effet à pH 7, que le tampon soit constitué de phosphate ou de Tris. Les ions magnésium, zinc et cadmium enfin, non seulement ne favorisent pas l'oxydation du sulfite, mais l'inhibent, comme l'indique le tableau II.

TABLEAU I

INFLUENCE DU pH SUR L'OXYDATION DU SULFITE PAR LE BROYAT DE RACINES OU PAR DES MÉTAUX, SOIT SEULS SOIT ADDITIONNÉS D'ULTRAFILTRAT DE BROYAT, OU D'ULTRAFILTRAT ET DE SÉRUM ALBUMINE

Sulfite 9.5 μmoles; broyat 0.1 ml (73 μg N total); métal 0.2 ml concentration finale $1 \cdot 10^{-5}\ M$; tampon Tris 0.05 M pH variable; ultrafiltrat 0.4 ml; sérum albumine 1 mg. Volume total 2 ml. Température, 37°. Résultats exprimés en μl O_2 consommés dans les 6 premières minutes.

pH	*5*	*5.6*	*6*	*6.2*	*6.5*	*7*	*7.3*	*7.6*	*8.1*
Broyat témoin	0	0	0	9	60	72	60	48	18
Cu^{++}	10	51	60	60	57	42	28	22	4
Cu^{++} et ultrafiltrat	0	0	0	0	2	3	3	2	1
Cu^{++} ultrafiltrat et sérumalbumine	0	0	0	0	1	2	2	1	1
Fe^{++}	0	4	6	9	8	6	5	5	1
Fe^{++} et ultrafiltrat	0	0	0	1	2	2	2	2	2
Fe^{++} ultrafiltrat et sérumalbumine	0	0	0	0	0	0	0	0	0
Mn^{++}	0	0	1	2	4	6	5	5	4
Mn^{++} et ultrafiltrat	0	0	0	0	1	4	4	4	0
Mn^{++} ultrafiltrat et sérumalbumine	0	0	0	0	0	4	5	4	0

TABLEAU II

INFLUENCE DU MAGNÉSIUM, DU ZINC ET DU CADMIUM SUR L'OXYDATION DU SULFITE PAR LE BROYAT DE RACINES D'AVOINE

Sulfite 9.5 μmoles, broyat 0.1 ml (30 μg N total) tampon phosphate 0.05 M pH 7.0. $MgCl_2$, $6H_2O$ $5 \cdot 10^{-4}\ M$; $Zn(CH_3–COO)_2$ $5.5 \cdot 10^{-4}\ M$; $Cd(CH_3–COO)_2$ $3.5 \cdot 10^{-4}\ M$. Température 37°, volume final 2 ml. Oxygène consommé exprimé en μl.

Conditions	*Temps (min)*			
	3	*6*	*12*	*20*
Broyat	18	27	35	41
Broyat + Mg^{++}	15	25	32	36
Broyat + Zn^{++}	6	9	14	16
Broyat + Cd^{++}	8	14	21	23

Influence des composés cétoniques

Partie expérimentale: On opère dans les conditions décrites dans la première section. On utilise 9.5 μmoles de sulfite et introduit 9.5 μmoles de pyruvate de sodium et 9.5 μmoles d'α-cétoglutarate de sodium. Dans ces essais on place 0.1 ml de potasse à 10% dans la cupule centrale des cellules de Warburg. Dans une autre série d'expériences, on met en présence les mêmes quantités de sulfite et de composés cétoniques à 37° et à pH 7, 40 min avant l'apport du broyat de racines (0.1 ml correspondent à 50 μg N total).

Résultats: En présence de broyat de racines d'avoine, ni le pyruvate ni l'α-cétoglutarate n'inhibent l'oxydation du sulfite, que l'on fasse le mélange en proportions équimoléculaires juste avant la mesure de l'oxydation ou qu'on laisse le sulfite et le composé cétonique en contact pendant 40 min. De plus, lorsque l'absorption d'oxygène atteint son maximum, il n'y a plus de sulfite décelable par iodométrie.

HANDLER[4], avec une préparation de foie de rat constate également que la présence de pyruvate ou d'α-cétoglutarate en proportion équimoléculaire avec le sulfite conduit à la même cinétique d'absorption d'oxygène que le sulfite seul. Au contraire TAGER[2], observe que le pyruvate à la concentration $5\cdot10^{-3}\,M$ et à pH 7.4 inhibe de 40 % la vitesse d'oxydation du sulfite ($4\cdot10^{-2}\,M$) en présence de mitochondries de feuilles d'avoine. TAGER suggère que cette inhibition est due à la formation d'un composé bisulfitique. Si cela est le cas, on doit admettre qu'en présence de broyat de racines la décomposition à pH 7 du complexe bisulfitique est au moins aussi rapide que l'oxydation du sulfite, ou que ce complexe peut être directement oxydé.

Influence des agents complexants

Partie expérimentale: On se place dans les conditions optima suivantes: sulfite 9.5 μmoles, broyat 0.1 ml correspondant à 74 μg d'azote total, tampon phosphate 0.05 M pH 7.0 et on introduit les substances indiquées:

Résultats: L'oxyde de carbone, les acides tartrique, citrique, sulfosalicylique et le fluorure de sodium n'inhibent pas aux concentrations utilisées l'oxydation du sulfite. Le cyanure au contraire (Fig. 4) est un inhibiteur. Une concentration en cyanure de $2\cdot10^{-5}\,M$ conduit à une diminution de 40 % de la consommation d'oxygène mesurée après 6 min. Avec une concentration 25 fois plus élevée, $0.5\cdot10^{-3}\,M$, on

Agents complexants du Fe^{+++}	*Concentration dans le milieu (M)*
Acide tartrique	$1\cdot10^{-3}$
Acide citrique	$1\cdot10^{-3}$
Acide sulfosalicylique	$0.5\cdot10^{-3}$
Fluorure de sodium	$2\cdot10^{-2}$
Cyanure de potassium	$2\cdot10^{-5}$
Cyanure de potassium	$0.5\cdot10^{-3}$
Versène	$0.5\cdot10^{-5}$
Versène	$1\cdot10^{-5}$
Versène	$2\cdot10^{-5}$
Versène	$4\cdot10^{-5}$

Agents complexants du Fe^{++}

Oxyde de carbone: atmosphère constituée de CO–O_2 (90:10) l'expérience est réalisée à 25° et à l'obscurité, en présence de témoins maintenus dans une atmosphère formée de N_2–O_2 (90:10).

o-phénanthroline	$2\cdot10^{-5}$
α,α'-dipyridyle	$2\cdot10^{-5}$

Agents complexants du Cu^{++}	
Pyrophosphate	$1\cdot10^{-3}$
Acide rubéanique	$1\cdot10^{-4}$
Acide thioglycollique	$2\cdot10^{-4}$
Acide thioglycollique	$2\cdot10^{-3}$
β-naphtol	$2\cdot10^{-4}$
β-naphtol	$2\cdot10^{-3}$
Hydroquinone	$2\cdot10^{-5}$
Hydroquinone	$1\cdot10^{-3}$
Hydroquinone	$5\cdot10^{-3}$
8-hydroxyquinoléine	$1\cdot10^{-5}$

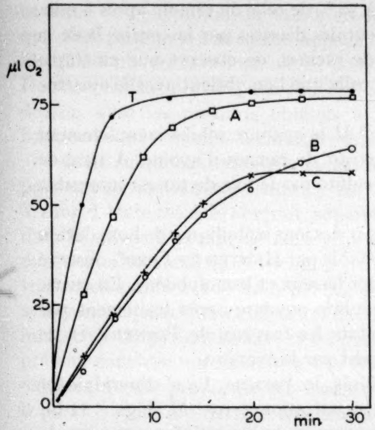

Fig. 4. Influence du cyanure de potassium sur l'oxydation du sulfite par le broyat de racines d'avoine. Sulfite, 9.5 μmoles; broyat, 0.1 ml (74 μg N total); tampon phosphate, 0.05 *M*; pH, 7.0. T, expérience témoin sans cyanure; A, KCN $2 \cdot 10^{-5}$ *M*; B, KCN $0.5 \cdot 10^{-3}$ *M*; C, KCN $0.5 \cdot 10^{-3}$ *M*, sans broyat.

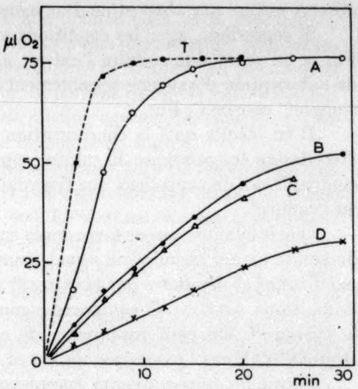

Fig. 5. Influence d'agents complexants du fer sur l'oxydation du sulfite par le broyat de racines d'avoine. Sulfite, 9.5 μmoles; broyat, 0.1 ml (74 μg N total); tampon phosphate, 0.05 *M*; pH, 7.0. Agents complexants à la concentration $2 \cdot 10^{-5}$ *M*. Volume, 2 ml; température, 37°. T, expérience témoin; A, cyanure de potassium; B, Versène; C, *o*-phénanthroline; D, *α*, *α'*-dipyridyle.

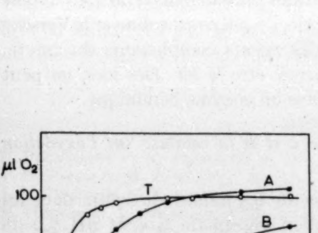

Fig. 6. Influence du Versène sur l'oxydation du sulfite par le broyat de racines d'avoine. Sulfite 9.5 μmoles; broyat, 0.1 ml (60 μg N total); tampon phosphate, 0.05 *M*, pH, 7.2; volume, 2 ml, température, 37°. T, expérience témoin; A, Versène $0.5 \cdot 10^{-5}$ *M*; B, $1 \cdot 10^{-5}$ *M*; C, $2 \cdot 10^{-5}$ *M*; D, $4 \cdot 10^{-5}$ *M*; E, $10 \cdot 10^{-5}$ *M*.

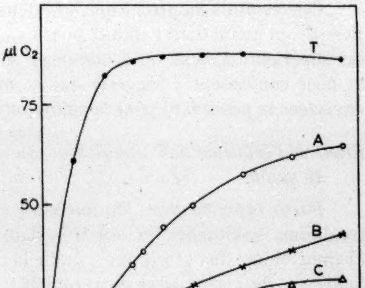

Fig. 7. Influence d'agents complexants du cuivre sur l'oxydation du sulfite par le broyat de racines d'avoine. Sulfite, 9.5 μmoles; broyat, 0.1 ml (74 μg N total); tampon phosphate, 0.05 *M*; pH, 7.0; volume final, 2 ml; température, 37°. T, expérience témoin; A, hydroquinone $1 \cdot 10^{-3}$ *M*; B, naphtol $2 \cdot 10^{-3}$ *M*; C, hydroquinone, $5 \cdot 10^{-3}$ *M*; D, thioglycollate de sodium $2 \cdot 10^{-3}$ *M*.

observe encore une absorption d'oxygène égale à 30 % de celle du témoin après 6 min.

Si cependant, avec les conditions expérimentales décrites par la courbe B de la Fig. 4 on omet d'introduire l'extrait aqueux de racines, on observe une cinétique de l'absorption d'oxygène sensiblement égale à celle que l'on obtient avec le système complet: courbe C, Fig. 4.

Il en résulte qu'à la concentration $0.5 \cdot 10^{-3}$ M le cyanure inhibe complètement l'oxydation enzymatique du sulfite propre à l'extrait de racines d'avoine. A titre de comparaison, on rappellera que l'oxydation du sulfite par le foie de rat est insensible au cyanure[4].

Que le cyanure puisse favoriser la catalyse par des ions métalliques de l'oxydation du sulfite est un phénomène signalé comme probable par HUNTER ET FORD[8], observé par TAGER[2] et démontré par HANDLER[9] avec le fer ferreux et le molybdène. En outre, la xanthine oxydase se comporte comme une sulfite oxydase après traitement par le cyanure[10]. On peut rapprocher de ces résultats les travaux de POSNER[11] et de BODLÄNDER[12] sur l'oxydation de l'or et de l'argent par le cyanure.

Parmi les autres agents complexants utilisés, le Versène, l'α,α'-dipyridyle et l'*o*-phénanthroline sont des inhibiteurs dès la concentration $2 \cdot 10^{-5}$ M (Figs. 5 et 6). Ce sont des chélateurs du fer. Les agents chélateurs du cuivre n'influencent pas la vitesse d'oxydation du sulfite à la concentration $2 \cdot 10^{-5}$ M, bien qu'ils en bloquent l'auto-oxydation en absence de broyat de racines. Mis à part le pyrophosphate qui n'est jamais inhibiteur, il faut augmenter la concentration de ces réactifs du cuivre jusqu'à une valeur de l'ordre de 10^{-3} M pour obtenir une inhibition de l'oxydation enzymatique du sulfite (Fig. 7).

Ces résultats montrent que le système enzymatique présent dans la racine d'avoine possède un métal dont l'affinité pour l'α,α'-dipyridyle, l'*o*-phénanthroline et le Versène est supérieure à celle qu'il manifeste à l'égard des agents complexants du cuivre, et nous conduisent à suggérer que ce métal pourrait être le fer. Dès lors, on peut envisager la possibilité pour la sulfite oxydase d'être un enzyme héminique.

Etude de l'influence de l'hémoglobine, du cytochrome c *et de la catalase sur l'oxydation du sulfite*

Partie expérimentale: On introduit en présence de 9.5 μmoles de sulfite dans les conditions habituelles, en solution dans du tampon phosphate 0.05 M pH 7.0 de l'hémoglobine, du cytochrome *c* ou de la catalase pures, de façon que la concentration finale soit de $1 \cdot 10^{-5}$ M et de $2 \cdot 10^{-5}$ M.

Résultats: Non seulement aucune de ces protéines héminiques ne catalyse l'oxydation du sulfite, mais elles inhibent l'auto-oxydation du sulfite au même titre qu'une protéine quelconque. On doit en conclure que la sulfite oxydase est un enzyme distinct des protéines héminiques usuelles.

CONCLUSION

L'extraction de racines de plantules d'avoine âgées de 5 jours par un tampon phosphate 0.1 M pH 7, contenant du chlorure de sodium à la concentration 0.35 M, permet d'obtenir une solution qui catalyse l'oxydation du sulfite en sulfate. Le pH optimum pour cette réaction est de 7 en tampon phosphate comme en tampon Tris 0.05 M.

Dans l'extrait aqueux étudié plusieurs substances peuvent contribuer à la catalyse de l'oxydation du sulfite en sulfate. L'une d'entre elles, et la plus active, que l'on peut provisoirement appeler sulfiteoxydase, est thermolabile, non ultrafiltrable à travers la cellophane, et possède un métal dont la nature n'est pas connue. Il est cependant possible que ce métal soit le fer, hypothèse qui n'est pas en contradiction avec les résultats obtenus à l'aide de divers agents chélateurs, ni avec l'aptitude de l'hématine à catalyser l'oxydation du sulfite en sulfate.

Cette sulfite oxydase de la racine d'avoine possède une activité catalytique qui, pour une concentration en sulfite $5 \cdot 10^{-3}\ M$, à pH 7 et à 37° conduit à un Q_{O_2} rapporté à 1 mg d'azote total de l'extrait, supérieur à 24,000. Le système enzymatique présent dans la racine d'avoine est ainsi plus actif que celui que TAGER a mis en évidence dans les mitochondries des feuilles d'avoine. Ce dernier en effet, pour une concentration en sulfite $2 \cdot 10^{-2}\ M$ à pH 7.4 et à 30°, possède un $Q_{O_{2N}}$ d'environ 100. Ces systèmes enzymatiques catalysant l'oxydation du sulfite se distinguent aussi par l'action inhibitrice du pyrophosphate et du pyruvate. Ces composés n'inhibent que l'enzyme présent dans ces mitochondries des feuilles. Cependant le cyanure inhibe la sulfite oxydase qu'elle provienne des feuilles, ou de la racine d'avoine. Quoiqu'il en soit il paraît prématuré de tirer des conclusions de ces comparaisons avant d'avoir des systèmes enzymatiques purifiés. Il paraît cependant certain que la sulfite oxydase des racines d'avoine est différente de celle, étudiée par HANDLER[4] dans les tissus des mammifères, qui en particulier n'est pas inhibée par le cyanure.

RÉSUMÉ

Il existe dans les racines d'avoine un système enzymatique catalysant l'oxydation du sulfite en sulfate. Le pH optimum pour le système enzymatique non purifié est de 7, en tampon phosphate. Les agents chélateurs de métaux sont en général des inhibiteurs.

BIBLIOGRAPHIE

[1] P. FROMAGEOT ET H. PEREZ-MILAN, *Biochim. Biophys. Acta*, 32 (1959) 457.
[2] J. M. TAGER ET N. RAUTANEN, *Biochim. Biophys. Acta*, 18 (1956) 111.
[3] J. M. TAGER ET N. RAUTANEN, *Physiol. Plant.*, 9 (1956) 665.
[4] M. HEIMBERG, I. FRIDOVICH ET P. HANDLER, *J. Biol. Chem.*, 204 (1953) 913.
[5] I. FRIDOVICH ET P. HANDLER, *J. Biol. Chem.*, 223 (1956) 321.
[6] E. ABEL, *Monatsh. Chem.*, 82 (1951) 815.
[7] E. C. FULLER ET R. H. CRIST, *J. Am. Chem. Soc.*, 63 (1941) 1644.
[8] E. E. HUNTER ET L. FORD, *Federation Proc.*, 13 (1954) 234.
[9] I. FRIDOVICH ET P. HANDLER, *Federation Proc.*, 15 (1956) 256.
[10] P. HANDLER ET I. FRIDOVICH, dans ROSCOFF, C. N. R. S., *Colloque sur la Biochimie du Soufre*, Paris, 1956, p. 83.
[11] L. POSNER, *J. Prakt. Chem.*, 34 (1846) 441.
[12] G. BODLÄNDER, *Angew. Chem.*, 19 (1896) 583.

AN ELECTROPHORETIC AND ULTRACENTRIFUGAL STUDY ON THE PROTEINS OF THE HIGH DENSITY FRACTION OF EGG YOLK*

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SUMMARY

1. The high density fraction represents 31% of the non-dialysable egg yolk solids. Electrophoretic analyses show that it contains six proteins in the proportions indicated below in order of decreasing mobility: phosvitin 15%; α-livetin 7%; β-livetin 17%; α-lipovitellin 30%; β-lipovitellin 21%; and γ-livetin 10%. No evidence was obtained to indicate the presence of a comparable proportion of any other protein.

2. Two lipoproteins that can be resolved electrophoretically, termed α- and β-lipovitellin, were found in the high density fraction. As another lipoprotein is present in the low density fraction, there are three lipoproteins in whole egg yolk, and these were demonstrated electrophoretically by adding a small proportion of the low density fraction to the high density fraction solutions.

3. Both α- and β-lipovitellin sediment as a single boundary in 1 M NaCl, but at higher pH values they dissociate to yield a slower sedimenting component. β-lipovitellin dissociates in a lower pH range than α-lipovitellin, but both appear to behave as reversible association–dissociation systems.

INTRODUCTION

Egg yolk proteins can be separated, on the basis of their density, into a HDF and a LDF[1]. The latter, previously referred to as the floating or rising fraction[2,3], is formed by a low density lipoprotein. The high density, or sedimenting, fraction contains five proteins: phosvitin[4,5], α-, β-, and γ-livetin[6,7], and lipovitellin[1,8]. In the present work electrophoretic and ultracentrifugal analyses were undertaken on HDF with a variety of solvents and under various experimental conditions, in an effort to resolve, identify, and estimate the relative proportions of its components.

In this investigation it was found that lipovitellin contains two electrophoretic components. In accordance with usual practice these have been called α- and β-lipovitellin. Their separation and characterisation will be reported in a later paper[9].

Abbreviations: HDF, high density fraction; LDF, low density fraction.

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METHODS

Concentration of solutions was determined primarily by sampling replicate aliquots of both solution and solvent after exhaustive dialysis, and drying these to a constant weight *in vacuo* at 60°. The specific refractive index increment (d*n*/d*c*) of the solute was then calculated from these gravimetric results, and routine concentration estimates made from the refractive index increments (Δn) of solutions as measured in a differential refractometer[10].

Sedimentation velocity measurements were made at 20° and 59,780 rev./min with conventional 12 or 30 mm cells in a Spinco Model E analytical ultracentrifuge equipped with temperature control. The boundary displacement was measured with a travelling microscope and sedimentation coefficients computed from plots of log x *vs.* t. These were corrected to standard conditions and extrapolated to zero concentration. When relative concentrations of different sedimenting components had to be estimated, double sector cells were used. Areas were measured on 9 × (linear) enlargements of the patterns with an Amsler mechanical integrator. No corrections were made for differences in the d*n*/d*c* of the components, for the JOHNSTON-OGSTON effect[11], or the dilution effect due to the sector shape of the cell.

Electrophoretic measurements were made in a Spinco Model H apparatus at 1°. Veronal buffer at pH 9.0 and 0.3 μ (containing 0.25 M sodium chloride) was the solvent used, unless otherwise stated. As the ionic strength necessary to keep the lipoprotein in solution was rather high, low voltage gradients were used to maintain a current of 16 mA, and most experiments extended for about 20 h. Boundary displacements were measured on the patterns of the descending limb with a travelling microscope and the mobilities computed from these values and the conductivity of the solvent at 0°. To estimate the relative proportions of the different components, area measurements were made with a mechanical integrator on enlargements (4 × linear) of the descending limb, where resolution of the components was more satisfactory. In a few instances electrophoretic components were isolated by means of a separation[12] or analytical cell.

PREPARATIONS

HDF

One volume of egg yolk, obtained by puncturing yolks from which any adhering white had been removed by rolling them on absorbent paper, was diluted to 3 volumes with 2 M NaCl and enough solid NaCl to bring the final concentration to 2 M NaCl. This suspension was stirred for 1 h at 4° and then centrifuged at 30,000 rev./min (No. 30 rotor) in a Spinco Model L preparative ultracentrifuge at 0° for 24 h. Four zones were then evident in the tubes (Fig. 1): A, a firm layer of yellow gel; B, a clear colorless solution; C, a viscous yellow solution grading to a firm pellet at the bottom; D, a fluffy yellow suspension. Layer A and the suspended material in layer D constitute LDF and the remainder HDF. Their separation was accomplished by breaking layer A and decanting layers B and D. The pellet C and adherent layer A were then separated by cutting the tube, and pellet C collected by washing with solution BD and then with the original solvent. When the pellet C had completely dissolved, the yellow suspension was centrifuged, as above, for 12 h and the small amount of LDF, originally present in suspension D, was removed from the top by suction. The remainder represents all the sedimenting material, termed HDF.

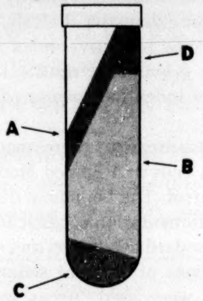

Fig. 1. Zones present in preparative ultracentrifuge tubes after high-speed centrifugation (24 h at 0° and 30,000 rev./min) of egg yolk diluted 1:3 with 2 M NaCl.

Fast sedimenting component (S_1)

The procedure was similar to that used to prepare HDF, except that both centrifugations were of 65-h duration. Only pellet C was retained after the first centrifugation. This was dissolved in the original solvent and centrifuged. Again only pellet C was retained after the second centrifugation.

Lipovitellin

Based on earlier observations[1,7,8], an extremely mild but effective procedure, outlined in Fig. 2, was developed for preparing lipovitellin, phosvitin, and the

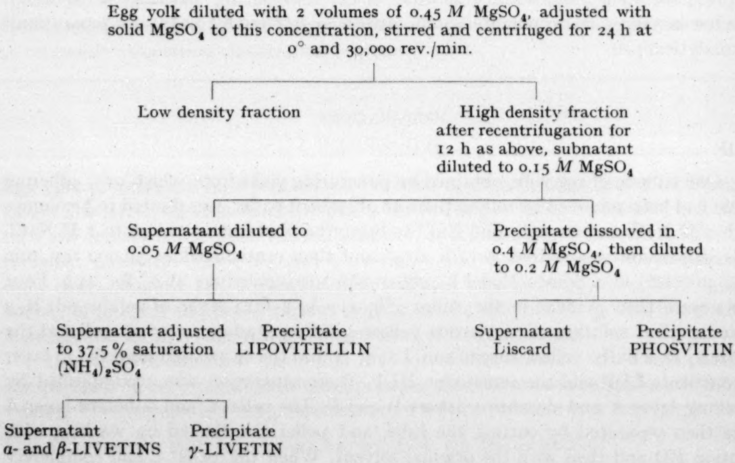
Egg yolk diluted with 2 volumes of 0.45 M MgSO4, adjusted with solid MgSO4 to this concentration, stirred and centrifuged for 24 h at 0° and 30,000 rev./min.
Low density fraction
High density fraction after recentrifugation for 12 h as above, subnatant diluted to 0.15 M MgSO4
Supernatant diluted to 0.05 M MgSO4
Precipitate dissolved in 0.4 M MgSO4, then diluted to 0.2 M MgSO4
Supernatant adjusted to 37.5 % saturation (NH4)2SO4
Precipitate LIPOVITELLIN
Supernatant Discard
Precipitate PHOSVITIN
Supernatant α- and β-LIVETINS
Precipitate γ-LIVETIN

Fig. 2. Procedure for the fractionation of HDF.

livetins. The HDF was first prepared as indicated above, except that 0.45 *M* $MgSO_4$ was used instead of 2 *M* NaCl. Two volumes of water were then added to the HDF drop-wise (0.15 *M* $MgSO_4$) with mechanical stirring in a room at 4°. After standing overnight, a yellow gelatinous precipitate adhered to the bottom of the flask. Resolution and precipitation of this fraction yielded phosvitin. The supernatant was decanted, diluted with two volumes of water (0.05 *M* $MgSO_4$), and allowed to stand overnight at 4°. The white precipitate of lipovitellin was recovered by centrifugation, dissolved in 1 *M* NaCl, and stored at 0°. This material was then dialysed exhaustively against the experimental solvent.

Addition of ammonium sulphate to the supernatant to 37.5 % saturation precipitated γ-livetin, while α- and β-livetin remained in solution.

Each step of the fractionation procedure was examined by ultracentrifugation, electrophoresis and total and protein phosphorus analysis of the fractions obtained. On this basis the indicated purity of each fraction was higher than 95 %. Impurities could be removed by reprecipitating each fraction by the method indicated in Fig. 2.

ELECTROPHORETIC RESULTS

When HDF was examined in veronal buffer at pH 9.0 and 0.3 μ, seven components were evident in the descending, and five in the ascending, limb (Fig. 3). Since the three leading boundaries in the descending limb were found subsequently to be phosvitin, these have been marked 1a, b and c. At pH 10.5 (0.2 μ glycine buffer) and at pH 8.5 (0.3 μ veronal) the patterns were similar to those at pH 9.0.

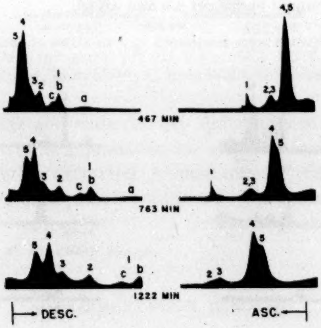

Fig. 3. Electrophoretic patterns of HDF in veronal pH 9.0, 0.3 μ.

In all buffers used, the shape of the patterns, the area and mobility of the components, differed in the ascending and descending limbs, a behavior that doubtless arises from the multiplicity of components of widely different charge density.

The electrophoretic components were identified by one or more of the following methods: (a) By adding isolated proteins to the HDF solution and comparing the patterns with the original material in parallel experiments; (b) by electrophoretic analyses of both the precipitates and supernatant solutions at each stage indicated in Fig. 2, supplemented by ultracentrifugal and chemical analyses; (c) by comparison of the mobilities of the HDF components with those of the individual proteins. These mobilities are reported in Table I.

Component 1 was identified as phosvitin by all three criteria. This peak was enhanced when phosvitin was added (Fig. 4A). The crude phosvitin precipitate obtained at 0.15 *M* $MgSO_4$ shows a minor amount of lipovitellin, but the main component has the characteristic pattern and mobility of phosvitin (Fig. 4B), and these fast peaks were absent from the supernatant. Purified phosvitin at this ionic strength was found to give three peaks (Fig. 4C) in the descending limb and one in the ascending, all with similar mobilities (Table I) to the leading peaks observed in HDF solutions.

TABLE I

ELECTROPHORETIC MOBILITY OF THE COMPONENTS OF THE HIGH DENSITY FRACTION*

Mobility of HDF component		*Mobility of protein isolated from HDF*	
*Component***	*cm²/sec/V × 10⁵*	*Protein*	*cm²/sec/V × 10⁵*
1a	— 10 to — 12	Phosvitin	— 10 to — 12
1b	— 8.5		— 8.0
1c	— 7.5		— 7.4
2	— 5.4	α-livetin	— 5.1***
3	— 3.7	β-livetin	— 3.4***
4	— 3.1	α-lipovitellin	— 3.2 (see ref. 9)
5	— 2.4	β-lipovitellin	— 2.7 (see ref. 9)
		γ-livetin	— 2.2

* All measurements in veronal buffer pH 9.0 and 0.3 μ.
** As indicated in Fig. 3.
*** Mobilities of α- and β-livetin were measured in a mixture of these two proteins.

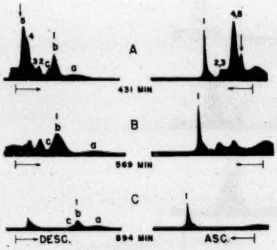

Fig. 4. Electrophoretic patterns in veronal pH 9.0, 0.3 μ of: A, HDF + phosvitin; B, precipitate obtained at 0.15 *M* $MgSO_4$ from HDF; C, phosvitin.

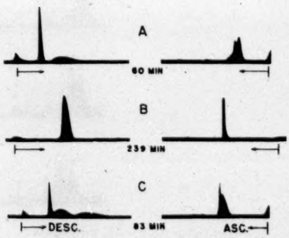

Fig. 5. Electrophoretic patterns of: A, phosvitin in veronal pH 9.0, 0.1 μ; B, phosvitin in veronal pH 9.0, 0.1 μ, + 0.01 *M* $MgSO_4$; C, phosvitin in veronal pH 9.0, 0.1 μ, + 0.005 *M* Na versenate.

This anomalous electrophoretic behavior of phosvitin was studied by determinations on purified material in veronal buffer at pH 9.0 and 0.1 μ (Fig. 5A) and in this buffer plus: 0.01 *M* $MgSO_4$ (Fig. 5B); 0.01 *M* $CaCl_2$ or 0.005 *M* sodium Versenate (Fig. 5C). At this lower ionic strength only two components were detectable in purified phosvitin; both magnesium and calcium complexes[5] were electrophoretically homogeneous; and the Versene-treated material had three components in the descending and two in the ascending, limb. Evidently the electrophoretic behavior of phosvitin depends on the ionic strength and bivalent cation content of the solution.

The identification of the livetins in the HDF pattern was undertaken by adding a 2:5 mixture of α- and β-livetin (containing a minor amount of γ-livetin). This enhanced peak 2 and, to a greater extent, peak 3 (Fig. 6A). The lipovitellin precipitate obtained at 0.05 *M* $MgSO_4$ (Fig. 2) was free of these components (Fig. 6B), while the peaks in the supernatant had the same mobilities as α-, β- and γ-livetin. This evidence indicated that components 2 and 3 (Fig. 3) are α- and β-livetin.

Components 4 and 5 must contain γ-livetin and lipovitellin. The precipitate at 0.05 *M* $MgSO_4$ (Fig. 6B), containing little or no γ-livetin, had two components of similar mobility and proportions to those in HDF (Table I). Evidently lipovitellin has two electrophoretic components, one of which is indistinguishable from γ-livetin at pH 9.0. Addition of γ-livetin enhanced component 5 (lowest mobility) in this buffer and at pH 10.6 (0.2 μ glycine buffer) it was possible to resolve added γ-livetin from the two lipovitellin boundaries (Fig. 6C).

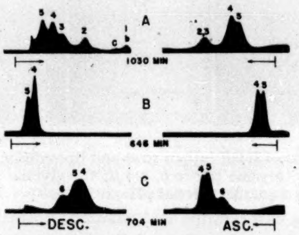

Fig. 6. Electrophoretic patterns in veronal pH 9.0, 0.3 μ of: A, HDF + α- and β-livetin; B, precipitate obtained at 0.05 *M* $MgSO_4$ from HDF; C, lipovitellin + γ-livetin in glycine pH 10.6, 0.2 μ.

The relative amounts and wide density differences of LDF and HDF made it impossible to maintain stable boundaries for electrophoretic analysis of whole egg yolk that had not been ether extracted. A small quantity of LDF could, however, be tolerated in the HDF veronal pH 9.0 solution. Upon electrophoresis LDF appeared as a component of still lower mobility than γ-livetin (Fig. 4A arrows) and was resolved (in the ascending limb) at an early stage.

SEDIMENTATION RESULTS

Ultracentrifugation in 1 *M* NaCl resolved HDF into two components termed S_1 and S_2[1], having $s^{\circ}_{20,w}$ values of 9.9 S (see ref. 9) and 3.4 S, (see ref. 6) respectively. All available evidence indicates that S_2 is formed by α- and β-livetin[6], so that S_1 must represent phosvitin, γ-livetin and lipovitellin, although its schlieren pattern (Fig. 7A1) suggests a monodisperse material.

When lipovitellin is prepared according to Fig 2, it also sediments in 1 *M* NaCl as a single component (Fig. 7A2) with $s^{\circ}_{20,w} = 10.5$ S (see ref. 9). This component is termed S_L to distinguish it from S_1 above.

Both S_1 and S_L have been shown to split into two components at pH 9.0, and, from its similar sedimentation properties, the minor component was taken to be

γ-livetin[8]. As this view was not consistent with information obtained in the present study, further investigations were undertaken. To avoid spurious effects of fractionation, the S_1 component was studied in the presence of S_2, as it occurs in HDF, and lipovitellin was prepared according to Fig. 2.

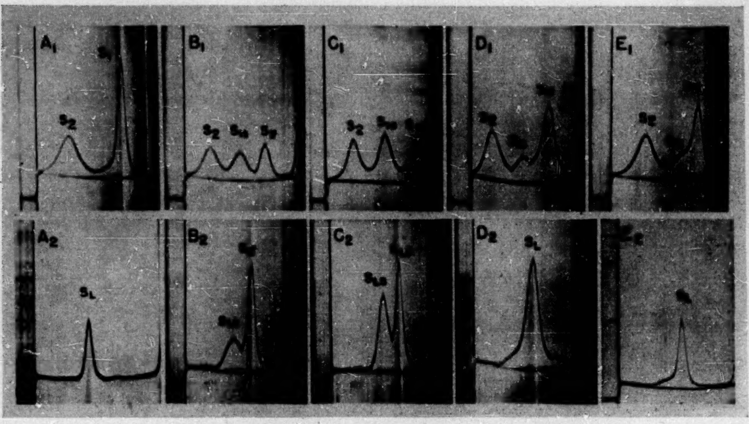

Fig. 7. Sedimentation patterns of HDF (upper row) and lipovitellin (lower row) in: A, 1 *M* NaCl; B, veronal pH 9.0, 0.3 μ; C_1, glycine pH 10.9, 0.2 μ; C_2, glycine pH 10.6, 0.2 μ; D, phosphate pH 6.8, 0.4 μ; E, veronal pH 9.0, 0.3 $\mu \longrightarrow$ 1 *M* NaCl.

The splitting that occurs when S_1 and S_L were examined in veronal at pH 9.0 is shown in Fig. 7B1 and 7 B2, respectively. The proportion of the slow components S_{1s} and S_{Ls} increased with increasing pH (Fig. 7C1 and C2), while S_2 remained constant in the HDF solutions. This confirms and extends an earlier observation[8] indicating that the major component, S_1, dissociates to an increasing extent as the pH is increased. In phosphate buffers pH 6.8 (0.4 and 0.8 μ) S_1 splits into two components (Fig. 7D1), but S_L does not (Fig. 7 D2). When solutions in veronal are dialysed into 1 *M* NaCl, S_{1s} remains in reduced amount and S_{Ls} practically disappears (Fig. 7 E). The proportions of S_{1s} and S_{Ls} components observed in these and other solvents are summarized in Table II.

The results in Table II show that there is about 20% more of a slow moving component in S_1 than in S_L. Presumably both S_1 and S_L are principally lipovitellin, which can dissociate into two components under certain conditions, especially at high pH. However, S_1 also contains approximately 20% of another slow moving component, which is either γ-livetin or an interaction product of γ-livetin and phosvitin.

Additional experiments were undertaken to obtain further information on the S_1 component. When this component, containing phosvitin, γ-livetin and lipovitellin, was prepared from HDF by centrifugation to remove S_2, and examined at pH 9.0, S_{1f} and S_{1s} were present, but phosvitin (3.5 S) was never evident. By diluting HDF directly from 0.45 *M* to 0.05 *M* $MgSO_4$ the precipitate contains lipovitellin and phosvitin, but no γ-livetin. This fraction sedimented as a single peak in 1 *M* NaCl,

TABLE II

PROPORTIONS OF SLOW SEDIMENTING COMPONENTS IN S_1 AND S_L, IN VARIOUS SOLVENTS

Solvent	*Ionic strength*	*pH*	S_{1s}*	S_{Ls}*
NaCl	1.0		0	0
Phosphate buffer	0.4	6.8	20	0
Phosphate buffer	0.8	6.8	18	0
Phosphate buffer	0.3	7.5	—	25
Veronal buffer	0.3	9.0	52	31
As above, dialysed back to NaCl	1.0		28	6
Veronal buffer	1.0	9.0	—	28
Glycine buffer	0.2	10.0	59	—
Glycine buffer	0.2	10.6	66	47
As above, dialysed back to NaCl	1.0		25	< 5
Glycine buffer	0.2	10.9	80	—

* Values are expressed as % of total S_1 or S_L.

but at pH 9.0 the 3.5 S boundary (phosvitin) was evident, in addition to the other two, and this 3.5 S boundary persisted when the preparation was dialysed back into 1 *M* NaCl. Evidently the removal of γ-livetin affects the sedimentation behavior of phosvitin.

The anomalous sedimentation behavior of γ-livetin, already reported[7], was again observed in veronal at pH 9.0 and 1 *M* NaCl solution. In sodium chloride solutions the *s vs. c* plot was positive, indicating aggregation with increasing concentration, although extrapolation yielded the reported[7] value of $s^\circ_{20,w} = 7.5$ S. In veronal at pH 9.0 the *s vs. c* plot gave the same intercept but was slightly negative. At $c = 0.5$ % the sedimentation coefficient in veronal was about 2 S units lower than in 1 *M* NaCl, and this behavior was reversible between these solvents. This indicates that failure to resolve γ-livetin from the S_1 component at finite concentration in 1 *M* NaCl may arise from its similar sedimentation rate rather than by interaction.

Composition of high density fraction

In order to express the proportions of the several components observed electrophoretically in HDF in terms of yolk solids, the proportion of HDF in yolk solids had to be determined. Since the components are dialysed before examination, the whole yolk solids were dialysed to permit their proportions to be expressed on a comparable basis. Evaporation and other factors affect the percentage solids found in egg yolk, but the loss on dialysis was about 2.5 %, and the non-dialysable solids were about 46.5 % of the total yolk contents. The separation of HDF (Fig. 2) was then performed quantitatively by using 2 *M* NaCl as solvent and somewhat more dilute solutions of yolk, washing the LDF, and adding these washings to the HDF. The amount of the starting material and HDF were determined by dry weight after exhaustive dialysis against sodium chloride solutions.

The results of replicated experiments, summarized in Table III, show that 31 ± 1 % of the non-dialysable yolk solids was HDF, and the LDF by difference about 69 %. As the electrophoretic separation of γ-livetin from the lipovitellin components in HDF was inadequate for measurement, the proportion of this protein was obtained from the content and patterns of the total livetins, separated from the other constituents on hydroxyapatite columns, to be described later[9].

TABLE III

PROPORTION OF PROTEINS IN HIGH DENSITY FRACTION BY ELECTROPHORETIC ANALYSIS

Component	As HDF %		As non-dialysable egg yolk solids %
HDF	100		31
Phosvitin	15		4.6
α-livetin	7		2.2
β-livetin	17		5.3
α-lipovitellin	30		9.3
β-lipovitellin	31	21	6.5
γ-livetin		10*	3.1

* As γ-livetin was not resolved in HDF, it was estimated from its electrophoretic proportion in the total livetins ($\alpha:\beta:\gamma = 2:5:3$) obtained by chromatographic separation[9].

DISCUSSION

Electrophoretic analysis of HDF solutions, conducted over long periods at low current densities in suitable solvents, revealed a maximum of seven boundaries in the descending limb. These were identified by electrophoretic, ultracentrifugal, and chemical analyses. It was found that the three boundaries of highest mobility were all given by phosvitin. The next two boundaries were given α- and β-livetin with mobilities in general agreement with previous reports[6]. These were followed by lipovitellin, which was found to have two electrophoretic components, termed α-lipovitellin and β-lipovitellin in order of decreasing mobility, as is the usual practice. Although γ-livetin was not resolved in HDF, it was found, on increasing its proportion by addition, that it had a lower mobility than either of the lipovitellins. This finding requires an earlier interpretation[6] of an electrophoretic pattern of whole egg yolk to be revised. With the recognition of α- and β-lipovitellin, all the electrophoretic components can be identified as proteins and lipoproteins previously isolated from HDF, and no evidence was obtained for the existence of other proteins in sufficient proportion to be detectable by the methods employed.

The α- and β-lipovitellins mentioned here are not the same as those described by SUGANO[13], which were prepared from ether extracted whole egg yolk and identified as lipovitellin and lipovitellenin, respectively. It was found here that the addition of LDF (not ether extracted) containing lipovitellenin to the HDF solutions gave a third lipoprotein boundary, the added LDF having the lowest mobility. SUGANO's lipovitellins differ both qualitatively and quantitatively from ours but, as he has found both his preparations to be heterogeneous mixtures ultracentrifugally[14], useful comparisons are impossible.

The several components of HDF cannot be separated quantitatively. Ultracentrifugation resolves only two boundaries, both containing two or more components. In consequence the best quantitative estimate of the concentration of the components was obtained from the electrophoretic patterns of HDF. As γ-livetin was not adequately resolved for satisfactory measurements, the proportion of the three livetins was estimated after their chromatographic separation[9] and found to be $\alpha:\beta:\gamma = 2:5:3$. Phosvitin represents about 15 % and the two lipovitellins about half of HDF. Since HDF was found to represent 31 % of the nondialysable yolk solids, this factor can be used to estimate their content in terms of yolk solids. As the protein content of the yolk solids is approx. 35 %, and HDF is approx. 31 %, the proportions of the proteins in HDF approximate, within experimental error, their proportions in the total lipid-free protein, if the lipid content of the lipovitellins is subtracted.

When HDF is dissolved in 1 *M* NaCl solutions, the lipovitellin, phosvitin and γ-livetin sediment as a single boundary, S_1 (see ref. 1, 8). At pH 9.0 S_1 splits and the slower minor boundary (termed S_{1s}) has the sedimentation properties of γ-livetin[7]. Lipovitellin behaves similarly, having a single boundary, S_L, in 1 *M* NaCl, and a slower component, S_{Ls}, at pH 9.0, originally regarded as a γ-livetin impurity[8]. Since lipovitellin, prepared according to Fig. 2, was free of phosvitin and γ-livetin by all criteria available, the origin of this second component was re-investigated. Comparison of HDF and lipovitellin solutions showed that S_{1s} was proportionately greater than S_{Ls} in a number of solvents, that both increased in quantity with increasing pH, and that only S_{Ls} disappeared completely when returned to 1 *M* NaCl. Evidently lipovitellin can dissociate reversibly into smaller units (S_{Ls}) under mildly alkaline conditions. The greater proportion of S_{1s}, and its incomplete reversibility on returning to 1 *M* NaCl, indicate that it contains two proteins that cannot be resolved ultracentrifugally. One of these is dissociated lipovitellin (S_{Ls}) and the other is γ-livetin or its interaction product with phosvitin. Recent measurements of the partial specific volume of S_{Ls} by an ultracentrifugal method[15] shows that it resembles lipovitellin in this property.

When the second component in S_1 at pH 9.0 was assumed as its γ-livetin content, the observed molecular weight of S_1 in sodium chloride solutions could not be harmonized with the molecular weight and apparent quantity of its components[8]. Since much of the second component is a dissociation product of lipovitellin that does not exist in sodium chloride solution, this inconsistency disappears. However, molecular weight estimates reported[8] for lipovitellin at pH 9.0 are likely to be somewhat low due to the presence of this dissociation product.

The relation between α- and β-lipovitellin and the association–dissociation products of lipovitellin observed ultracentrifugally was examined by recovering the separated components from the electrophoretic cell for sedimentation analyses. At pH 9.0 α-lipovitellin sedimented as a single S_L boundary, whereas β-lipovitellin sedimented as a mixture of approx. 80 % S_{Ls} and 20 % S_{Lf}. Since most of the total lipovitellin dissociates at pH 10.9, it is evident that α-lipovitellin also dissociates under more alkaline conditions.

In earlier studies[8] it was found that the content of the S_{Ls} component at pH 9.0 could be reduced following certain precipitation procedures. In the present study repeated precipitation at 0.1 *M* $MgSO_4$ yielded a sample that contained only 5 % S_{Ls}. Electrophoretic examination also showed that the precipitate obtained on dilution to 0.1 *M* $MgSO_4$ contained less β-lipovitellin than the precipitate obtained at 0.05 *M* $MgSO_4$. Repeated precipitation could therefore fractionate the two components.

The separation of the two electrophoretic components[9] and quantitative measurements on these and their dissociation products will be reported later.

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REFERENCES

[1] J. E. Vandegaer, M. E. Reichmann and W. H. Cook, *Arch. Biochem. Biophys.*, 62 (1956) 328.
[2] K. J. Turner and W. H. Cook, *Can. J. Biochem. and Physiol.*, 36 (1958) 937.
[3] W. G. Martin, K. J. Turner and W. H. Cook, *Can. J. Biochem. and Physiol.*, 37 (1959) 1197.
[4] D. K. Mecham and H. S. Olcott, *J. Am. Chem. Soc.*, 71 (1950) 3670.
[5] F. J. Joubert and W. H. Cook, *Can. J. Biochem. and Physiol.*, 36 (1958) 399.
[6] W. G. Martin, J. E. Vandegaer and W. H. Cook, *Can. J. Biochem. and Physiol.*, 35 (1957) 241.
[7] W. G. Martin and W. H. Cook, *Can. J. Biochem. and Physiol.*, 36 (1958) 153.
[8] F. J. Joubert and W. H. Cook, *Can. J. Biochem. and Physiol.*, 36 (1958) 389.
[9] G. Bernardi and W. H. Cook, *Biochim. Biophys. Acta*, 44 (1960) 96.
[10] B. A. Brice and M. Halwer, *J. Opt. Soc. Am.*, 41 (1951) 1033.
[11] J. P. Johnston, A. C. Ogston, *Trans. Faraday Soc.*, 42 (1946) 789.
[12] R. A. Kekwick, J. W. Lyttleton, E. Brewer and E. S. Dreblow, *Biochem. J.*, 49 (1951) 253.
[13] H. Sugano, *J. Biochem. (Tokyo)*, 45 (1958) 393.
[14] H. Sugano, *J. Biochem. (Tokyo)*, 46 (1959) 417.
[15] W. G. Martin, C. A. Winkler and W. H. Cook, *Can. J. Chem.*, 37 (1959) 1662.

SEPARATION AND CHARACTERIZATION OF THE TWO HIGH DENSITY LIPOPROTEINS OF EGG YOLK, α- AND β-LIPOVITELLIN*

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SUMMARY

1. The electrophoretic components of lipovitellin (α- and β-) have been separated from each other, and from the livetins and phosvitin in the high density fraction of egg yolk, by chromatography on hydroxyapatite columns.

2. Both α- and β-lipovitellin have the same lipid content, nitrogen content, amino acid composition, and molecular weight ($4.0 \cdot 10^5$). They differ in their protein phosphorus content, electrophoretic mobility, absorption on hydroxyapatite, ultracentrifugal behaviour in alkaline media, and in solubility.

INTRODUCTION

It was shown in a recent paper[1] that the HDF of egg yolk contains two lipoproteins, α- and β-lipovitellin, differing in electrophoretic mobility. In addition, their sedimentation behaviour shows that both dissociate into smaller entities, β-lipovitellin being dissociated at pH 9.0 and α-lipovitellin at higher pH values. As the separation of

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Abbreviation: HDF, high density fraction; LDF, low density fraction.

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these lipovitellins by preparative electrophoresis[1] was considered inadequate for further work, their separation was undertaken by column chromatography. The separation of α- and β-lipovitellin and their characterization by chemical and physical means are described in this paper.

METHODS

Total nitrogen and sulphur were determined by micro-Kjeldahl and micro-Carius, respectively.

Phosphorus was estimated either by ALLEN's method[2] or the method of BERENBLUM AND CHAIN[3], depending on the phosphorus content of the sample.

Total lipids were extracted from 1–1.5 g amounts of material as approx. 10 % solutions in 1 *M* sodium chloride by 20 volumes of ethanol–ether (3:1)[4] at room temperature. The protein precipitate was removed by centrifugation, washed twice with ethanol–ether, and the combined extracts concentrated to a small volume on a rotary evaporator under reduced pressure. A large volume of ethyl ether was then added to precipitate the contaminating sodium chloride. Ethyl ether was used instead of petroleum ether because the latter is known to dissolve sodium chloride in the presence of phospholipids[5]. The sodium chloride was removed by centrifugation, washed twice with ethyl ether, centrifuged again, and the supernatant and washings concentrated, as above. The extract was then transferred quantitatively into weighing flasks, evaporated under a nitrogen stream and finally dried to a constant weight *in vacuo* at 40°.

Phospholipids were separated from the non-phospholipids by chromatography of the total lipids on silicic acid columns[6] using the method of LEA, RHODES AND STOLL[7]. The first fraction (non-phospholipids) was eluted with chloroform containing 2 % methanol, and the second (phospholipids) with methanol. Both were estimated gravimetrically. The recovery from the column was quantitative. The phosphorus content of the total lipids, multiplied by a factor of 25, was also used to estimate the amount of phospholipids[8].

Chromatographic separations of the proteins of the HDF of egg yolk were made at 4° on columns of hydroxyapatite by the method of TISELIUS, HJERTÉN AND LEVIN[9], using a time-operated fraction collector. A few experiments were done with 1 × 25 cm columns using small amounts of HDF to obtain an elution curve. The O.D. of these fractions were measured in a Beckman DU spectrophotometer at 280 mμ using 1 cm silica cells. When the O.D. was greater than 1.0, silica plungers were used to reduce the depth of solution. The large-scale separations were performed on 2.5 × 90 cm columns at a flow rate of 40 ml/h. About 3 g proteins were generally applied to the column as about 5 % solutions in 0.2 *M* potassium phosphate buffer pH 6.8, which was the lowest concentration that would maintain the lipovitellin in solution. Three elution steps were generally used, namely, 0.2 *M*, 0.6 *M*, and 2 *M* potassium phosphate. The high protein concentration of the eluates made further concentration unnecessary. The eluates were examined by electrophoretic and ultracentrifugal methods.

Amino acid analyses were carried out using the technique of MOORE, SPACKMAN AND STEIN[10]. The effluent was analysed according to MOORE AND STEIN[11]. Lipoprotein samples, previously defatted by alcohol–ether[5], were hydrolysed by refluxing with redistilled 6 N HCl for 22 h. The determination of cysteine–cystine as cysteic acid was done on samples oxidized with performic acid[12], dried *in vacuo*, and then hydrolysed, as above. The molar ratio of tryptophan and tyrosine was determined in 0.1 N NaOH with a Carey Model 11 recording spectrophotometer[13]. This ratio and the tyrosine content obtained by column chromatography were used to estimate the tryptophan content.

The protein concentration of the solutions, sedimentation velocity and electrophoretic mobility were measured by the methods previously described[1]. Partial specific volumes ($\bar{v}$) were determined at 20° in a magnetic float equipment[14], and here the concentrations were measured as weight fractions.

Molecular weight estimates were made by ARCHIBALD's method[15,16] at speeds between 4133 and 8225 rev./min, depending on the material investigated. Measurements were made only at the meniscus, since the use of either carbon tetrachloride or Dow Corning No. 515 silicone fluid to locate the base of the cell[17] caused a progressive broadening of the air-solution meniscus, possibly due to a piling-up of lipids set free from the lipoprotein. Protein concentrations of 0.8–1.0 % were generally used for these determinations, and the enlarged patterns (8 × linear) were measured with a mechanical integrator and computed according to SMITH, WOOD AND CHARLWOOD[18].

Diffusion coefficients were measured at 20° by either the Rayleigh fringe or the schlieren cylindrical lens method. The interference patterns were analysed according to LONGSWORTH's method[19]. The diffusion coefficients by the schlieren method were measured either in a Spinco Model H apparatus (avoiding any movement of the cell turret) or in a synthetic boundary cell (valve or capillary type) in the ultracentrifuge operated at 4133 or 8225 rev./min, and computed by the height–area method.

The HDF, and its major sedimenting component (S_1), were prepared by the sedimentation method, and lipovitellin by fractional precipitation from magnesium sulphate solutions, as previously described[1].

Chromatographic separation

Preliminary chromatographic experiments showed that no separation of the egg yolk proteins was obtained on brushite[9], but hydroxyapatite columns, prepared according to TISELIUS *et al.*[9], effectively fractionated HDF. Hydroxyapatite may also be useful for fractionating the LDF, since fractions were obtained from it at 0.3 and 0.7 M phosphate buffer pH 6.8 in initial trials, but the possibility of "false" components[20] was not investigated with LDF.

The elution curve obtained when HDF was chromatographed on a column of hydroxyapatite is shown in Fig. 1. The first fraction left the column without being absorbed at the buffer molarity required to dissolve the material (0.2 M). Ultracentrifugal examination of this fraction in veronal buffer pH 9.0 and 0.3 μ showed only the S_2 component (α- and β-livetin) and γ-livetin[1]. Electrophoretic analysis (Fig. 2A) also showed that it was made up of the three livetins in the proportions $\alpha:\beta:\gamma = 2:5:3$. When this first fraction is rechromatographed, it again leaves the column at 0.2 M potassium phosphate buffer. Preliminary experiments indicate that the three livetins can be fractionated on hydroxyapatite columns at phosphate buffer concentrations between 0.05 and 0.15 M.

The second chromatographic component, obtained with 0.6 M phosphate buffer, was evidently β-lipovitellin, since it was largely dissociated when examined ultracentrifugally at pH 9.0 (see ref. 1). Electrophoretic analysis showed that it was contaminated with a minor amount of α-lipovitellin, which was removed on rechromatographing (Fig. 2B).

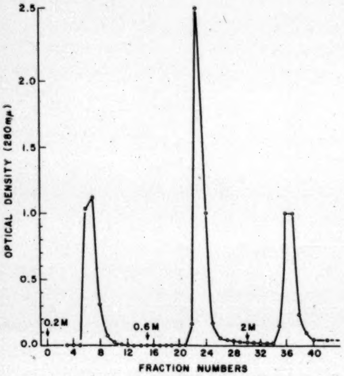

Fig. 1. Chromatography of HDF on hydroxyapatite.

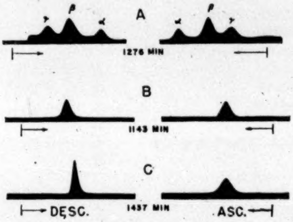

Fig. 2. Electrophoretic patterns in veronal buffer pH 9.0, 0.3 μ of HDF fractions obtained by column chromatography: A, total livetin fraction; B, α-lipovitellin; C, β-lipovitellin.

The third chromatographic fraction obtained with 2 *M* phosphate buffer was evidently α-lipovitellin contaminated with a small amount of β-lipovitellin, which was removed on rechromatographing (Fig. 2C). Phosvitin was retained on the column and did not appear in any of the eluates, as indicated by electrophoretic and other analyses. This behaviour is in accordance with the reported affinity of polyphosphates for hydroxyapatite[9].

Results of physical measurements

The results of the physical measurements on the S_1 component of HDF, prepared by the sedimentation procedure already described[1], lipovitellin and its α- and β-components, are summarized in Table I. The electrophoretic mobilities of isolated α- and β-lipovitellin in veronal buffer (pH 9.0, 0.3 μ) are in good agreement with their individual mobilities in the mixture obtained by preparing lipovitellin by precipitation.

Most of the measurements were made in 1 *M* NaCl to avoid the dissociation of lipovitellin in alkaline solvents of lower ionic strength. Small but consistent differences in the sedimentation coefficients of α- and β-lipovitellin suggest minor differences in volume or shape. The molecular weights obtained from the sedimentation and diffusion coefficients and the transient state method[15], however, are the same for α- and β-lipovitellin and their parent mixture within experimental error. As expected, the molecular weight of lipovitellin is somewhat higher than that previously observed* in dissociating solvents. The molecular weight of the S_1 component will be discussed later.

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* Through a computation error involving the magnification factor, the diffusion coefficient reported earlier for lipovitellin[21] in veronal buffer (pH 9.0, 0.3 μ) was too high and M too low. The correct values are: $D^{0}_{20,w} = 3.26 \cdot 10^{-7}$ and $M = 3.7 \cdot 10^5$. Likewise, for S_1 in 1 *M* NaCl[21], $D = 2.68 \cdot 10^7$, $M = 4.1 \cdot 10^5$; and for phosvitin in acetate buffer (pH 4.0, 0.1 μ)[22], $D = 4.60 \cdot 10^7$ and $M = 3.6 \cdot 10^4$.

TABLE I

SUMMARY OF PHYSICAL MEASUREMENTS

Property	*Solvent**	*S_1 component*	*Lipovitellin*	*α-lipovitellin*	*β-lipovitellin*
Electrophoretic mobility (10^{-5} cm²/sec/V)	B		−3.2 (α) −2.8 (β)	−3.2	−2.7
dn/dc (concentration in g/ml) at 5780 Å, 25°	A	0.179	0.181	0.180	0.180
v (ml/g) at 20°	A	0.765**	0.778	(0.777)***	0.777
$S°_{20,w}$ (Svedbergs)	A	9.9	10.5	10.9	10.4
	B		11.9§	12.0	
ds/dc (concentration in g %)	A	−0.75	−0.35	−0.50	−0.40
	B		−1.80§	−1.02	
$D_{20,w}$ (10^{-7} cm²/sec) at concentration given					
Rayleigh	A	2.5 (0.28 %)			
Schlieren					
Electrophoresis cell			2.9 (0.33 %)		
Ultracentrifuge				3.0 (0.47 %)	2.8 (0.75 %)
Molecular weight (× 10^5)					
from s and D	A	4.1	4.0	4.0	4.0
from ARCHIBALD's method §§	A	4.0 (0.93 %)	4.0 (0.94 %)	3.8 (0.47 %)	3.8 (1.00 %)

* Solvent A was 1 M NaCl; solvent B veronal buffer pH 9.0, 0.3 μ (0.25 M NaCl).

** At 25°.

*** Assumed value based on lipovitellin and β-lipovitellin.

§ In solvent B values given are for major component, minor component had $S°_{20,w}$ = 7.4 S and ds/dc = −0.30.

§§ Not corrected for concentration dependence. Correction would have increased reported value by 5 % or less.

The ultracentrifugal behaviour of α- and β-lipovitellin in neutral and alkaline solvents, together with comparable studies on the S_1 component and lipovitellin, supplementing earlier results[1], are shown in Fig. 3. In 1 M NaCl the S_1 component (Fig. 3A1), lipovitellin (Fig. 3A2), α-lipovitellin (Fig. 3B1) and β-lipovitellin (Fig. 3B2) all form single, sharp and symmetrical boundaries. In veronal buffer (pH 9.0, 0.3 μ) the slower sedimenting component is barely evident in α-lipovitellin (Fig. 3C1), whereas in β-lipovitellin (Fig. 3C2) this slow component represents about 80 % of the material. In glycine buffer (pH 10.9, 0.2 μ) α-lipovitellin (Fig. 3D1) dissociates to about the same extent as β-lipovitellin (Fig. 3D2), the degree of dissociation of the latter being about the same as at pH 9.0.

RESULTS OF CHEMICAL ANALYSES

The results of the chemical analyses made on lipovitellin and its α- and β-components are given in Table II. Lipovitellin and its separated components all contain about 20 % lipid, made up of 12 % phospholipid in terms of the total lipoprotein, and the remainder as triglycerides, cholesterol etc. The differences found between the reported lipid content and composition of the different lipovitellins are of doubtful significance.

Analysis of the two vitellins (*i.e.*, defatted lipovitellins) indicates that they have the same nitrogen content and tyrosine/tryptophan ratio. They differ markedly in phosphorus content and to a lesser extent in sulphur content. The latter difference

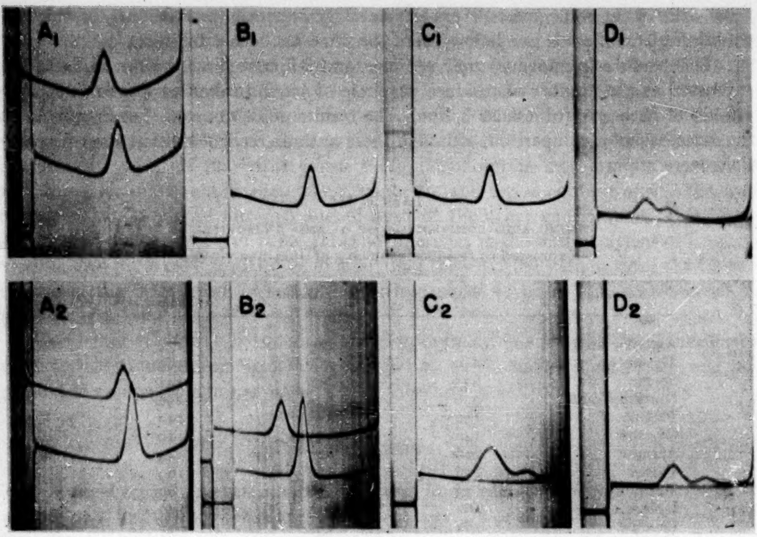

Fig. 3. Sedimentation patterns of: A_1, S_1 component in 1 M NaCl; A_2, lipovitellin in 1 M NaCl; B_1, α-lipovitellin in 1 M NaCl; B_2, β-lipovitellin in 1 M NaCl; C_1, α-lipovitellin in veronal buffer pH 9.0, 0.3 μ; C_2, β-lipovitellin in veronal buffer pH 9.0, 0.3 μ; D_1, α-lipovitellin in glycine buffer pH 10.9, 0.2 μ; D_2, β-lipovitellin in glycine buffer pH 10.9, 0.2 μ.

TABLE II

SUMMARY OF CHEMICAL ANALYSES*

Constituent		*Lipovitellin*	*α-lipovitellin*	*β-lipovitellin*
Total lipids	%	19	(20)**	20
Phospholipids				
(lipid P × 25)	%	12.5	11.0	13.5
(chromatography)	%	11.6		12.4
Non-phospholipids				
(chromatography)	%	7.4		7.6
Total phosphorus	%	1.05	1.40	0.95
Lipid phosphorus	%	0.50	0.44	0.54
As % of lipid	%	2.63	2.20	2.70
Protein phosphorus	%	0.50	0.96	0.36
As % of vitellin		0.62	1.20	0.45
Protein nitrogen	%		13.76	13.72
As % of vitellin	%		17.20	17.15
Protein sulphur	%		1.15	0.96
As % of vitellin	%		1.44	1.20
Tyrosine/tryptophan				
molar ratio		3.54	3.84	3.85

* Reported values are expressed as percent of lipoprotein, except as otherwise indicated.
** Assumed value.

must arise from some over-all experimental error since the amounts of sulphur-containing amino acids (see below) were the same for both vitellins.

The results of amino acid analyses on α- and β-lipovitellin are given in Table III, expressed as the number of residues per mole of vitellin taken as $3.2 \cdot 10^5$, *i.e.*, lipovitellin of $M = 4.0 \cdot 10^5$ less 20 % lipid. The results of LEWIS *et al.*[23] are reported on the same basis for comparison, although these authors recognized that their preparations were impure.

TABLE III

AMINO ACID COMPOSITION OF α- AND β-LIPOVITELLIN

Expressed as residues/320,000 g of lipid-free vitellin.

Constituent	*α-vitellin*	*β-vitellin*	*"Vitellin" of* LEWIS *et al.*[23]
Aspartic acid	273	267	194
Threonine*	149	160	126
Serine*	255	260	341
Glutamic acid	326	334	239
Proline	156	155	122
Glycine	140	132	119
Alanine	226	217	144
Valine	178	188	169
Methionine	73	76	60
Isoleucine	162	175	129
Leucine	263	257	210
Tyrosine	95	88	67
Tryptophan**	25	23	17
Phenylalanine	91	92	77
Lysine	161	169	151
Histidine	61	58	62
Arginine	153	162	154
Cystine/2***	58	56	40
Total amino acid N	3612	3663	3175
Total N §	3929 (17.20 %)	3917 (17.15 %)	3587 (15.7 %)
Amide N §§	(317)	(254)	295
P §	124 (1.20 %)	46 (0.45 %)	227 (2.2 %)
S §	144 (1.44 %)	120 (1.20 %)	99 (0.99 %)

* Corrected for hydrolysis by extrapolating values after 24 and 72 h hydrolysis to zero time.
** From tyrosine–tryptophan ratio (by u.v. spectroscopy) and tyrosine content.
*** As cysteic acid after performic acid oxidation, corrected by assuming 82 % recovery[24].
§ Computed from values in Table II and LEWIS *et al.*[23].
§§ Values in parentheses are difference between total protein and amino acid nitrogen.

Most of the amino acids in α- and β-vitellin do not differ in amount by more than 4 % and none by more than 8 %. In some hydrolysates of both vitellins, the aspartic and glutamic acid contents were about 10 % lower than those reported. A satisfactory explanation for this behaviour is lacking but, tentatively, it is suggested that the presence of any residual lipid may have caused some destruction of these acidic amino acids, and the higher values are taken to be more reliable.

The reported values for half-cystine have been corrected for 82 % recovery[24]. The moles of sulphur-containing amino acids found in both vitellins are in good agreement and suggest a sulphur content between those (Table II) found for α- and β-vitellin, respectively. from elementary analysis.

The serine and threonine values were corrected for losses during hydrolysis by extrapolating the figures obtained after 24 and 72 h hydrolysis back to zero time. These corrections indicated about 20 % destruction of serine in both vitellins during the 22-h hydrolysis period used. It is noteworthy that the moles of serine exceed the moles of phosphorus present in both fractions.

When the amino acid composition of α- and β-vitellin is compared with that reported for impure vitellin[23], it is evident that phosvitin was the main impurity. Since about 50 % of phosvitin consists of phosphoserine residues[23], its presence as a contaminant would increase the phosphorus and serine content of vitellin, and decrease the amount of nitrogen and of most of the other amino acids. This effect is indicated by the results in Table III. The difference in the serine content of LEWIS's vitellin, and ours, may actually be greater than the reported values, since the former estimated the destruction of serine during hydrolysis to be 10 %, a value which is probably too low.

A further similarity between α- and β-lipovitellin is suggested by a similarity of their N-terminal amino acids[25]. Lysine and an as yet unidentified amino acid are present in comparable proportions in both vitellins.

DISCUSSION

The physical measurements reported here and in an earlier paper[1] give a consistent picture of α- and β-lipovitellin and the parent mixture of these two components. The two electrophoretic components have been separated chromatographically and found to have the same molecular weight in 1 *M* NaCl. Small but consistent differences in the sedimentation coefficients suggest a slight difference in volume or shape. Both α- and β-lipovitellin dissociate into smaller sub-units when dissolved in alkaline solvents and reassociate when returned to 1 *M* NaCl. β-lipovitellin dissociates at lower pH values than α-lipovitellin, but neither is completely dissociated at pH 10.9. No attempt was made to obtain quantitative estimates of the sizes of the sub-units from the measurements made on these paucidisperse systems at pH 10.9, but estimates based on the frictional ratios, and other known properties of α- and β-lipovitellin, and the sedimentation coefficients of the sub-units suggest that the latter are half the size of their parent molecules. This has been confirmed in other solvents (4 *M* urea) that cause complete dissociation, and will be reported later[26].

Obviously, the sub-units from α-lipovitellin cannot both be identical with those of β-lipovitellin, since these two lipoproteins differ in phosphorus content, but the available evidence is too limited to determine whether the sub-units of each lipovitellin are identical or different. Similarity of the sub-units of β-lipovitellin is suggested by its electrophoretic homogeneity in veronal buffer at pH 9.0 and 0.3 μ, a solvent in which 80 % of this lipoprotein is dissociated. The presence of at least two N-terminal amino acids in β-vitellin, however, indicates that either the sub-units are different or that each contains more than one polypeptide chain.

The only chemical difference found between α- and β-lipovitellin lies in their protein phosphorus content. Contamination arising from several sources may affect their apparent phosphorus content. The affinity of phosvitin for hydroxyapatite and the electrophoretic homogeneity of the separated α- and β-lipovitellin give assurance that the difference in protein phosphorus content is not due to the presence of phosvitin. Another possibility is that the lipovitellin fractions retain some phosphate from the eluent buffer. All samples were dialysed exhaustively against 1 M NaCl on removal from the column and the only analyses reported in Table II are those in which the phosphorus content of the diffusate (control) was zero. Some earlier analyses, in which the control sample was positive, showed a smaller differential in the phosphorus content of α- and β-vitellin, after subtracting the control value. In all instances, however, the minimum value for α-vitellin always exceeded the maximum value for β-vitellin. Therefore, even if the quantities reported are subject to revision on further study, there is little doubt that the two vitellins differ in phosphorus content. Their electrophoretic and chromatographic behaviour support this conclusion.

The observed molecular weight of S_1 ($4.1 \cdot 10^5$) in 1 M NaCl substantiates earlier conclusions on the nature of this component. In this solvent S_1 is a mixture of: a predominating amount of undissociated α- and β-lipovitellins, an associated form of γ-livetin and phosvitin. While γ-livetin, in its associated form, may have a molecular weight lower than the lipovitellins, its effect on the average molecular weight is offset by the interaction of phosvitin with one or more of the other proteins. The net result is a mixture having the physical constants of an entity with a molecular weight that is the same as that of the major lipovitellin components.

From previous electrophoretic studies[1] it was indicated that the two lipovitellins are present in the proportions $\alpha:\beta = 3:2$. When lipovitellin is prepared by the precipitation procedure, it is evidently fractionated to some extent, and the proportions present after successive precipitations indicate that β-lipovitellin is more soluble than α-lipovitellin. Consequently, the proportions of the two lipovitellins in preparations obtained by precipitation may not represent their ratios in HDF. This unavoidable fractionation of precipitated lipovitellin, and some uncertainty about the precise phosphorus contents of α- and β-vitellin, also preclude a valid estimate of their proportions in HDF based on their protein phosphorus content. In principle, an independent estimate of their proportions could be obtained from the chromatographic elution curve. Integration of the O.D. of the individual fractions obtained on elution indicated a 1:1 ratio. The sharp separation observed in the small-scale experiments, however, may be only apparent, since the material had to be rechromatographed to effect complete separation in the large-scale work. This possibility, and the variation in the fraction volumes with different density eluents, make the electrophoretic evaluation of the α-:β-lipovitellin ratio in HDF the most reliable.

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REFERENCES

[1] G. BERNARDI AND W. H. COOK, *Biochim. Biophys. Acta*, 44 (1960) 86.
[2] R. J. L. ALLEN, *Biochem. J.*, 34 (1940) 858.
[3] I. BERENBLUM AND E. CHAIN, *Biochem. J.*, 32 (1938) 295.
[4] W. R. BLOOR, *J. Biol. Chem.*, 17 (1914) 377.
[5] H. N. CHRISTENSEN, *J. Biol. Chem.*, 129 (1939) 531.

[6] B. Borgstrom, *Acta Physiol. Scand.*, 25 (1952) 101.
[7] C. H. Lea, D. N. Rhodes and R. D. Stoll, *Biochem. J.*, 60 (1955) 353.
[8] J. C. Hawke and C. H. Lea, *Biochem. J.*, 54 (1953) 479.
[9] A. Tiselius, S. Hjertén and Ö. Levin, *Arch. Biochem. Biophys.*, 65 (1956) 132.
[10] S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, 30 (1958) 1185.
[11] S. Moore and W. H. Stein, *J. Biol. Chem.*, 211 (1954) 907.
[12] E. Schram, S. Moore and E. J. Bigwood, *Biochem. J.*, 57 (1954) 33.
[13] G. H. Beaven and E. R. Holiday, *Advances in Protein Chem.*, 7 (1952) 320.
[14] P. A. Charlwood, *J. Am. Chem. Soc.*, 79 (1957) 776.
[15] W. J. Archibald, *J. Phys. and Colloid Chem.*, 51 (1947) 1204.
[16] S. M. Klainer and G. Kegeles, *J. Phys. Chem.*, 59 (1955) 952.
[17] A. Ginsburg, D. Appel and H. K. Schachman, *Arch. Biochem. Biophys.*, 65 (1956) 545.
[18] D. B. Smith, G. C. Wood and P. A. Charlwood, *Can. J. Chem.*, 34 (1956) 364.
[19] L. G. Longsworth, *J. Am. Chem. Soc.*, 74 (1952) 4155.
[20] S. Hjertén, *Biochim. Biophys. Acta*, 31 (1959) 216.
[21] F. J. Joubert and W. H. Cook, *Can. J. Biochem. and Physiol.*, 36 (1958) 389.
[22] F. J. Joubert and W. H. Cook, *Can. J. Biochem. and Physiol.*, 36 (1958) 399.
[23] J. C. Lewis, N. S. Snell, D. J. Hirschmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, 186 (1950) 23.
[24] J. R. Kimmel, E. O. P. Thompson and E. L. Smith, *J. Biol. Chem.*, 217 (1955) 151.
[25] J. M. Neelin, personal communication.
[26] G. Bernardi and W. H. Cook, *Biochim. Biophys. Acta*, 44 (1960) 105.

MOLECULAR WEIGHT AND BEHAVIOR OF LIPOVITELLIN IN UREA SOLUTIONS*

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SUMMARY

Lipovitellin in 4 *M* urea has a molecular weight of $2.0 \pm 0.2 \cdot 10^5$, which is half that observed in 1 *M* NaCl. The two sub-units present in urea are undistinguishable on the basis of their ultracentrifugal properties. β-Lipovitellin dissociates at lower urea concentrations than α-lipovitellin. Although lipovitellin is denatured by urea and suffers a loss of lipid in this solvent, its dissociation is partly reversible on reducing the concentration of urea.

INTRODUCTION

Recent studies[1,2] have shown that lipovitellin and its two electrophoretic components (α- and β-lipovitellin) all sediment as a single component in 1 *M* NaCl, and have a molecular weight of $4.0 \cdot 10^5$ in this solvent. In alkaline solvents, however, lipovitellin partially dissociates to yield a second slower sedimenting component[1]. A monodisperse solution of these sub-units is required to estimate their molecular weight but, since

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complete dissociation was not obtained at pH values as high as 10.9 (see ref. 2), alkaline solvents were considered unsatisfactory. Since lipovitellin has been shown[3] to dissociate completely into a slower sedimenting component in 4 *M* urea solutions, this solvent was used in the present study to estimate the molecular weight of the sub-units..

METHODS AND MATERIAL

The physical and chemical methods employed have been described in earlier papers[1, 2]. In determining total lipids[2] this fraction was re-extracted with chloroform, as urea is less soluble in this solvent than in ethyl ether. Diffusion measurements in urea solutions were limited to those with schlieren optics in a Spinco Model H apparatus or in a synthetic boundary cell in the ultracentrifuge[2], as the high refractive index of 4 *M* urea solutions precluded the use of interferometric methods. Several attempts to adjust the optical paths lead to distorted fringes. All physical measurements were made at 20°.

Lipovitellin was prepared by the magnesium sulphate method[1] and α- and β-lipovitellin by chromatographing the high density fraction of egg yolk on hydroxyapatite columns[2]. Most of the measurements were made on lipovitellin prepared by precipitation, since α- and β-lipovitellin have the same molecular weight as the unfractionated material, and the sub-units cannot as yet be separated. Purity of these preparations was confirmed by sedimentation and electrophoretic analyses.

Lipovitellin and its α- and β-components were adjusted to the urea concentrations required by dialysing the starting solution (in 1 *M* NaCl or phosphate buffer, respectively) against urea solutions containing either 0.25 *M* NaCl or veronal buffer (pH 9.0 and 0.3 μ).

RESULTS

The conversion of lipovitellin to the slower component was essentially complete in 24 h in solvents containing 4 *M* urea (Fig. 1B). At lower urea concentrations two components were evident (Fig. 1A), and the quantity of the slower sedimenting component increased slightly with time over several days. Exposure to solvents containing 4 *M* urea for periods of one month caused only a slight decrease in the sedimentation coefficients of the slow component. No differences were detected in the behaviour of samples in urea–veronal (pH 9.0) and in urea–0.25 *M* NaCl.

When 4 *M* urea was diluted by dialysis to 2 *M* urea, a fast sedimenting component was again evident (Fig. 1C). This indicates partial reversibility, although the proportion of fast component is somewhat less than in material exposed only to 2 *M* urea (Fig. 1A). On further reduction of the urea concentration to 1.5 *M*, a partial precipitation of lipovitellin occurred. Essentially complete precipitation occurred when the urea was entirely removed by dialysis against veronal buffer (pH 9.0), originally a good solvent for lipovitellin. This loss of solubility and the formation of gels when the protein concentration exceeded about 3 % show that lipovitellin is denatured by 4 *M* urea.

Only a few experiments were made with separated α- and β-lipovitellin. β-lipovitellin is largely dissociated in 1 *M* urea (Fig. 1D) and the fast component had almost disappeared in 2 *M* urea (Fig. 1E). The fast component, however, still predominates

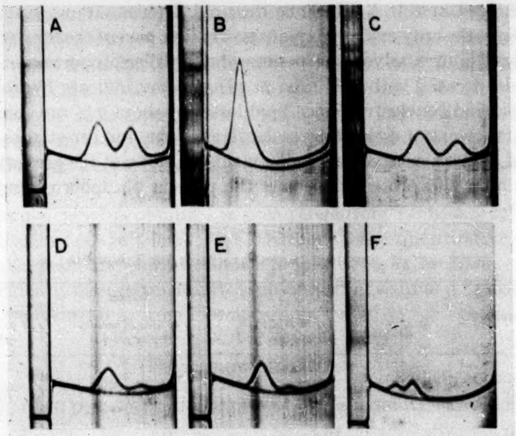

Fig. 1. Sedimentation patterns, in double sector cell at 50,740 rev./min, selected to show resolution in the different solvents: A, lipovitellin in 2 M urea + 0.25 M NaCl; B, lipovitellin in 4 M urea + 0.25 M NaCl; C, lipovitellin in 4 M urea + 0.25 M NaCl as dialysed back to 2 M urea + 0.25 M NaCl; D, β-lipovitellin in 1 M urea + 0.25 M NaCl; E, β-lipovitellin in 2 M urea + 0.25 M NaCl; F, α-lipovitellin in 2 M urea + 0.25 M NaCl.

in α-lipovitellin at this urea concentration (Fig. 1F). The relative degree of dissociation of the two lipovitellin fractions at different urea concentrations therefore parallels their behaviour with increasing pH values[2].

Electrophoretic analyses in 4 M urea were inconclusive because it was difficult to maintain stable boundaries. In a few experiments two boundaries were evident.

The results of the chemical analyses are summarized in Table I for lipovitellin that had not been treated with urea[2], and for material in 4 M urea. The lipovitellin dissolved in urea solutions contained only 12 % total lipid, as compared with 19 %

TABLE I

SUMMARY OF CHEMICAL ANALYSES ON LIPOVITELLIN*

Constituent		*1 M NaCl** pH approx. 7.0*	*4 M urea in veronal buffer, pH 9.0*	*4 M urea in 0.25 M NaCl, pH approx. 7.0*
Total lipids	%	19	12	12
Phospholipids				
(lipid P × 25)	%	12.5	6.75	9.00
(chromatography)	%	11.6	7.1	8.3
Non-phospholipids				
(chromatography)	%	7.4	4.9	3.7
Total phosphorus	%	1.05	0.85	
Protein phosphorus	%	0.50	0.52	
As % of vitellin		0.62	0.59	
Lipid phosphorus	%	0.50	0.27	0.36
As % of lipid		2.63	2.25	3.00

* Reported values are expressed as percent of lipoprotein, except as otherwise indicated.
** From BERNARDI AND COOK[2].

in samples dissolved in 1 *M* NaCl. Since the lipid determinations were made on the dialysed solutions, the only evident explanation is that part of the lipid was lost during the initial dialysis in urea solvents. The somewhat smaller proportion of phospholipid found in samples treated with 4 *M* urea at pH 9.0 may indicate hydrolysis but this cannot be considered conclusive. Since lipid losses of about 7 % are comparable with the experimental errors of estimating molecular weight, further studies on the effect of urea on lipid content were considered beyond the scope of the present work. While urea solutions alter the protein molecule, the protein phosphorus content was not affected.

TABLE II

SUMMARY OF PHYSICAL MEASUREMENTS ON LIPOVITELLIN

Property	*Solvent*		
	*1 M NaCl** *pH approx. 7.0*	*4 M urea in veronal buffer pH 9.0*	*4 M urea in 0.25 M NaCl pH approx. 7.0*
d*n*/d*c* (concentration in g/ml) at 5780 Å, 25°	0.181	(0.198)**	0.200
v (ml/g)	0.778	(0.737)**	0.744
$s^\circ_{20,w}$ (Svedbergs)	10.5	6.2	5.8
d*s*/d*c* (concentration in g %)	—0.35	—0.19	—0.33
$D_{20,w}$ (10^{-7} cm^2/sec)			
Schlieren			
In electrophoresis cell	2.9 (0.33 %)	2.8 (0.33 %)	—
In ultracentrifuge		3.2 (→ 0)	—
d*D*/d*c* (concentration in g %)		0.5	—
Molecular weight (× 10^5)			
From *s* and *D*	4.0	2.0	—
		1.8	—
From ARCHIBALD's method***	4.0	2.1 (0.70 %)	2.2 (0.73 %)

* From BERNARDI AND COOK[2].
** From JOUBERT AND COOK[3].
*** Not corrected for concentration dependence.

The physical measurements are summarized in Table II, including the comparable figures for lipovitellin that had not been exposed to urea. The higher specific refractive increment (d*n*/d*c*) and lower partial specific volume ($\bar{v}$) of lipovitellin in urea, as compared with values obtained in sodium chloride solutions, confirm previous observations[3]. The diffusion coefficients are only slightly higher than that of lipovitellin in 1 *M* NaCl. Molecular weights of 1.8 to $2 \cdot 10^5$ were computed from these coefficients. Slightly higher values obtained by ARCHIBALD's transient state method, not corrected for concentration dependence, were confirmatory.

DISCUSSION

Lipovitellin dissociates in urea solutions in a manner that parallels its dissociation in alkaline solvents[1,2]. Although urea solutions denature lipovitellin, the dissociation in this solvent is also partly reversible, as the faster sedimenting component reappears on diluting 4 *M* to 2 *M* urea. Again, β-lipovitellin, which dissociates more completely than α-lipovitellin at low pH values, shows the greatest degree of dissociation at low urea concentrations. Evidently most of the slow component of unfractionated lipovitellin in 2 *M* urea is β-lipovitellin.

While the sub-units obtained in alkaline and urea solvents may have the same origin, the sedimenting entities are not identical. A loss of lipid occurs in urea solutions and this may result from the formation of urea adducts of the fatty acids. Actually, the reported chemical analyses are compatible with the loss of one fatty acid molecule from each molecule of triglyceride or phospholipid. The sedimentation coefficient ($s^{\circ}_{20,w}$) of the sub-units is 7.4 S in alkaline solution[2] and only approx. 6.0 S in urea, a difference that can be explained by the changes in the protein moiety caused by urea denaturation.

The molecular weights obtained in urea range from 1.8 to $2.2 \cdot 10^5$, which is half that of lipovitellin ($4.0 \cdot 10^5$) in *M* NaCl. Although the quantitative measurements cannot be made with the same accuracy in 4 *M* urea as in dilute buffer solution, lipovitellin evidently dissociates into two sub-units that sediment as a single boundary, but may not be identical in size or composition.

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REFERENCES

[1] G. Bernardi and W. H. Cook, *Biochim. Biophys. Acta*, 44 (1960) 86.
[2] G. Bernardi and W. H. Cook, *Biochim. Biophys. Acta*, 44 (1960) 105.
[3] F. J. Joubert and W. H. Cook, *Can. J. Biochem. and Physiol.*, 36 (1958) 389.

STUDIES ON THE PLASMALOGENS OF NERVOUS TISSUE

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SUMMARY

The concentration and distribution of plasmalogens in various regions of the human nervous system and in nervous and other tissues of some animal species have been investigated.

Only traces of choline plasmalogen were detected in any region of the human or animal nervous systems examined. About 50 % of the ethanolamine phosphatide of grey matter and probably all or nearly all that of white matter and spinal cord is present as plasmalogen.

INTRODUCTION

The total plasmalogen content of nervous tissue has been studied by a number of authors in recent years. Stammler, Stammler and Debuch[1] and Rapport and

LERNER[2] have investigated some regions of the human nervous system, while studies on brain in various animal species have been reported by CHRISTL[3], MINDER AND ABELIN[4] and WITTENBERG, KOREY AND SWENSON[5]. FOLCH-PI[6] and more recently KOREY AND ORCHEN[7], DAVISON AND WAJDA[8] and ERICKSON AND LANDS[9] have studied the variations in plasmalogen content of whole brain as a function of age in animals.

A lipid mixture extracted from whole ox brain was found to contain only very little choline plasmalogen[10]; it was further found that the plasmalogen in the cephalin fraction of whole brain is present mainly as the ethanolamine type, only small amounts being present in the serine phosphatide fraction[11,12].

Although it is now well recognised from these previous studies that white matter contains considerably more plasmalogen than grey matter, little information seems to be available concerning possible variations in the amounts of the different types of plasmalogen lipid, or in the proportion of ethanolamine phosphatide present in the plasmalogen form, in the various regions of the central and peripheral nervous system. In view of increasing interest in these lipids and lack of knowledge concerning their biological significance, it seemed desirable to investigate these points. During the development of procedures suitable for the study of the biochemistry and metabolism of the aldehydogenic moiety of the plasmalogens some information on these questions was obtained and is presented below. Similar data on a few non-nervous tissues are also included for comparison.

EXPERIMENTAL

Materials

Human nervous tissues were obtained post-mortem as soon as possible after death.

Ox brain was obtained from the slaughter house from freshly killed animals.

Monkey spinal cord was removed from the animal immediately after death and deep-frozen for several days before extracting the lipids.

Rats and hens were killed by decapitation and the tissues to be examined removed immediately.

Separation of lipids

The lipids were extracted from the tissues by homogenization in a Waring blendor in 19 volumes chloroform–methanol (2:1, v/v) and the extracts, after filtration, washed with $^1/_5$ their volume of water as described by FOLCH, LEES AND SLOANE-STANLEY[13]. Aliquots of the washed extracts containing 0.3–2.0 mg lipid phosphorus were evaporated to dryness in vacuo at 60° and the lipids separated on columns of alumina[14]. 5 g of alumina (Hopkin and Williams, "Activated, for chromatography") were used for each column; the columns used were 7 cm long and 1 cm in diameter. $CHCl_3$-insoluble protein, which separated from proteolipid during evaporation of the solvent from the washed total lipid extract, was filtered off on the column. Lipids were eluted with a succession of solvents according to the technique of LONG described by DAVISON AND WAJDA[8].

Four fractions were obtained by elution with (a) 20 ml $CHCl_3$, (b) 30 ml chloroform–methanol (1:1, v/v), (c) 15 ml chloroform–methanol–water (7:7:1, v/v/v) and (d) 30 ml chloroform-ethanol–water (2:5:2, v/v/v). These four fractions contained respectively (a) cholesterol, (b) choline phosphatide (lecithin plus sphingomyelin), (c) cerebroside and (d) cephalin.

In preliminary experiments with rat brain lipids it was found that the fraction eluted with $CHCl_3$ was free of phosphorus and fuchsin-positive material. In the above procedure slight tailing of the choline phosphatide occurred in the final stages of the elution with chloroform–methanol (1:1, v/v) and traces of phosphorus (3–4 % of the initial column loading) were present in the cerebroside fraction; a finding in agreement with that of DAVISON AND WAJDA[8]. Very slight traces of plasmalogen, but no ninhydrin-positive material were detected in the cerebroside fraction, which was therefore combined with the second fraction from the columns for the determination of choline plasmalogen and phosphorus.

Analytical methods

Phosphorus was determined by the method of ERNSTER, ZETTERSTRÖM AND LINDBERG[15] as modified by STRICKLAND, THOMPSON AND WEBSTER[16]. Plasmalogen was determined by the modification of the method of LEUPOLD AND BUTTNER[17] described by GRAY AND MACFARLANE[18]; pure dimethyl acetal of palmitaldehyde (kindly provided by Dr. G. M. GRAY) was used as a standard. Ethanolamine and serine were estimated by the fluorodinitrobenzene method of AXELROD, REICHENTHAL AND BRODIE[19], following hydrolysis of the lipid samples in 6 *N* HCl in sealed tubes for 3 h at 100°. Since the high water content of the cephalin fraction caused considerable foaming if the solvents were evaporated directly, the water and methanol contents were reduced by washing aliquots of this fraction with 2/3 their volume of 0.1 *N* NaCl, two phases then formed and were separated by centrifuging. The phospholipids were recovered quantitatively from the lower $CHCl_3$-rich phase.

Solvents: Chloroform and methanol (Hopkin and Williams, AR) and ethanol (99.4 % Burrough, RR Grade) were used as supplied.

RESULTS

Recoveries of lipids from alumina columns

The total recoveries of lipid phosphorus and plasmalogen in the second and fourth fractions eluted from the columns during chromatography of lipid extracts of various nervous tissues are shown in Table I.

It can be seen that except in the case of human sciatic nerve 75–87 % of the lipid phosphorus applied to the columns was recovered in these fractions. The recovery of lipid phosphorus of human sciatic nerve was rather less (70 %).

The total amount of plasmalogen recovered in these two fractions from human and rat central nervous tissues was 91–103 % of the initial column loading. The recovery of plasmalogen from human sciatic nerve and all the hen nervous tissues studied was, however, less complete (79–87 %).

In a more detailed study of the cephalin fractions from some human and rat nervous tissues it was found that only traces of serine were present in this fraction, which, however, contained 81–91 % (mean 88 %) of the ethanolamine present in the total lipid extracts applied to the columns. It seems likely, therefore, that the incomplete recovery of lipid phosphorus from the column may be due largely to failure to elute the serine phosphatide component of the cephalins.

TABLE I

RECOVERY OF LIPIDS FROM ALUMINA COLUMNS

Sum of amounts of lipid phosphorus and plasmalogen in choline phosphatide (sphingomyelin plus lecithins) and cephalin fractions, expressed as percentage of amounts applied to the column.

Species	*Tissue*	*% recovery*	
		P	*Plasmalogen*
Human	Cortex	78	101
	Putamen	78	101
	Internal capsule	78	95
	Corpus callosum	75	95
	Spinal cord	78	103
	Sciatic nerve	70	79
Hen	Cerebrum	87	87
	Spinal cord	82	80
	Sciatic nerve	81	82
Rat	Cerebrum	79	96
	Spinal cord	81	91

Nervous tissue

The values found for total lipid phosphorus and plasmalogen contents in various regions of the human nervous system are set out in Table II. The choline plasmalogen content, expressed as a percentage of the total, is also shown.

The total amount of plasmalogen in central white matter and in the spinal cord is 3–4 times that found in the cortex or putamen, and the percentage of phospholipid present as plasmalogen in the white matter (30–36 %) was between one and a half and two times that found in grey matter. In peripheral nerve the plasmalogen content of the total phospholipids resembled that of white matter and spinal cord.

TABLE II

PLASMALOGEN OF HUMAN NERVOUS TISSUES

Tissue	*µmoles/g fresh tissue*		*B as % of A*	*Choline plasmalogen as % of B*
	Total lipid P (A)	*Total plasmalogen (B)*		
Cortex	47.0	9.2	20	1.5
	44.5	7.2	16	1.7
Putamen	64.3	13.4	21	2.1
	57.4	9.4	17	3.2
Internal capsule	108.3	32.8	30	3.0
	123.8	40.6	33	3.4
	84.8	30.8	36	3.4
Corpus callosum	108.3	32.9	30	2.9
	99.6	34.5	35	3.5
Spinal cord	88.3	29.6	34	2.6
	104.9	35.9	34	3.7
Sciatic nerve	37.6	13.9	37	3.6
	54.2	16.8	31	3.3

The results found for nervous tissues from various animal species are shown in Table III. Spinal cord in both the hen and the rat contained about twice the amount of plasmalogen present in human cord (Table II). On a molar basis approx. 50% of the phospholipid in the spinal cords of these species was in the form of plasmalogen.

TABLE III

PLASMALOGEN OF ANIMAL NERVOUS TISSUES

Species	*Tissue*	*µmoles/g fresh tissue*		*B as % of A*	*Choline plasmalogen as % of B*
		Total lipid P (A)	*Total plasmalogen (B)*		
Hen	Cerebrum	58.0	15.0	26	2.0
		67.2	16.7	25	1.2
		60.6	14.4	24	1.2
	Spinal cord	111,2	63.9	57	2.4
		149.0	67.6	45	2.5
		140.0	64.5	46	2.4
	Sciatic nerve	77.7	31.2	40	1.3
		71.6	24.9	35	1.0
		65.4	26.3	40	0.8
		79.0	34.3	40	1.7
Rat	Cerebrum	79.0	22.6	29	2.8
		80.0	25.9	32	2.4
		79.0	20.6	26	2.1
		70.0	18.7	27	2.2
		70.4	17.7	25	2.3
		71.7	18.0	25	1.9
	Spinal cord	127.8	64.4	50	3.7
		140.8	64.2	46	4.8
Ox	Cortex	59.0	10.3	17	2.4
	Internal capsule	125.0	37.0	30	3.5
	Medulla	169.0	52.6	31	2.5
Monkey	Spinal cord	120.0	43.4	36	2.4

TABLE IV

LIPID PHOSPHORUS AND PLASMALOGEN OF CEPHALIN FRACTIONS OF NERVOUS TISSUES RECOVERED FROM ALUMINA COLUMNS

Each figure is the mean of at least two experimental results.

Species	*Tissue*	*µmole/g fresh tissue*		*Molar ratios P: Plasmalogen*
		P	*Plasmalogen*	
Human	Cortex	17.1	8.2	1 : 0.48
	Putamen	22.0	11.6	1 : 0.52
	Internal capsule	39.0	33.0	1 : 0.87
	Corpus callosum	35.4	31.0	1 : 0.88
	Spinal cord	36.1	32.8	1 : 0.90
	Sciatic nerve	13.4	11.7	1 : 0.87
Hen	Cerebrum	26.5	13.1	1 : 0.50
	Spinal cord	56.7	50.8	1 : 0.90
	Sciatic nerve	28.8	23.1	1 : 0.80
Rat	Cerebrum	27.4	16.8	1 : 0.62
	Spinal cord	59.8	55.8	1 : 0.93

It is evident from Tables II and III that only traces of choline plasmalogens could be detected in any of the regions of human or animal nervous systems studied.

The results of analyses of the cephalin fractions obtained from human, rat and hen nervous tissues for lipid phosphorus and plasmalogen are shown in Table IV. It can be seen that in the cephalin fractions from various regions of human brain rich in white matter and also in those from spinal cord and peripheral nerve in all three species, 87–93 % of the phospholipid was present in the plasmalogen form. In human cerebral cortex and putamen, on the other hand, only 48 and 52 % respectively of the phospholipid was present as plasmalogen. The value for whole rat cerebrum (62 %) was intermediate between these values for human grey and white matter.

In view of these differences between grey and white matter experiments were carried out with some human and rat nervous tissue to obtain more detailed information concerning the composition of the cephalin fractions. The molar ratios of lipid phosphorus to ethanolamine in these fractions from the tissues examined

TABLE V

ANALYSIS OF CEPHALIN FRACTIONS OF NERVOUS TISSUES RECOVERED FROM ALUMINA COLUMNS

Species	*Tissue*	*µmole/g fresh tissue*				*Molar ratios*
		P	*Plasmalogen*	*Ethanolamine*	*Serine*	*P: Plasmalogen: Ethanolamine: Serine*
Human	Cortex	17.2	7.5	14.7	0.8	1 : 0.44 : 0.85 : 0.05
	Internal capsule	32.3	29.3	29.1	0.5	1 : 0.91 : 0.90 : 0.01
	Corpus callosum	36.8	31.7	35.5	0.5	1 : 0.86 : 0.96 : 0.01
Rat	Cerebrum	27.6	16.8	24.8	1.8	1 : 0.61 : 0.90 : 0.07
		27.1	16.9	25.7	2.3	1 : 0.62 : 0.95 : 0.08
	Spinal cord	59.8	55.4	55.7	4.2	1 : 0.93 : 0.93 : 0.07
		59.8	56.2	57.7	3.1	1 : 0.94 : 0.96 : 0.06

TABLE VI

PLASMALOGEN OF NON-NERVOUS TISSUES

Species	*Tissue*	*µmoles/g fresh tissue*		*B as % of A*	*Choline plasmalogen as % of B*
		Total lipid P (A)	*Total plasmalogen (B)*		
Rat	Heart	33.0	4.4	13	21
		34.0	5.3	14	19
		40.0	3.8	10	12
	Skeletal muscle	19.0	2.9	15	13
		18.5	2.0	11	16
	Kidney	58.5	4.1	7	10
		61.1	4.8	8	11
	Liver	45.0	1.0	2	19
		55.0	0.7	1	25
Hen	Heart	43.8	7.5	17	40
		32.1	7.9	25	46
	Skeletal muscle	9.1	2.8	31	34
		11.6	3.5	30	40
	Liver	36.7	1.1	3	14
		32.2	0.9	3	7

were between 1:0.85 and 1:0.96, while only traces of serine were present. These results are set out in Table V.

Non-nervous tissues

The results of analyses of a small number of non-nervous tissues from the rat and the hen, shown in Table VI, are in general agreement with the findings reported by other workers for the tissues of various animal species. With the exception of hen heart the total amount of plasmalogen in these tissues is considerably less than that of nervous tissues. Liver contains only traces in both species.

Hen heart contains 1.5–2.0 times as much as rat heart, but the values for skeletal muscle are similar in the two species. The percentage of plasmalogen in the total phosphatide was also found to be rather higher in heart and approximately twice as high in skeletal muscle of the hen as compared with the rat. The choline plasmalogen content of heart and skeletal muscle is also considerably greater in the hen than in the rat.

DISCUSSION

In general, in earlier studies on plasmalogen content of tissues the values reported have referred to the wet or dry weight of the tissue. In the present study lipid phosphorus has been used in addition to wet weight as a standard of reference and when expressed in this way the results show good agreement with those of RAPPORT AND LERNER[2].

The results for peripheral nerve, which even after careful dissection still contains an appreciable proportion of connective tissue, show that the proportion of plasmalogen in the total phospholipid is closely similar to that of central white matter and spinal cord in both the hen and man.

The total plasmalogen content of hen heart is comparable to that of the rabbit[2] and the ox[18]. The relatively smaller amount in rat heart would seem to be due at least partly to its lower choline plasmalogen content. The possible significance of this species difference and of the greater proportion of plasmalogen in rat and hen cord as compared with human cord, is obscure because of our ignorance of the biological role of plasmalogens in tissues.

With regard to the composition of the cephalin fractions obtained from nervous tissues the data given in Tables IV and V show that this probably consists of about 85–95 % ethanolamine phosphatide accompanied by traces only of serine phosphatide. Nearly all the plasmalogen and 81–91 % of the ethanolamine phosphatide of the tissues examined was recovered in this fraction. DAVISON AND WAJDA (personal communication) have shown, in similar studies with rabbit brain, that the inositol phosphatide is quantitatively recovered from alumina columns in the cephalin fraction. The amount of inositol phosphatide in brain is, however, less than $^1/_{10}$ that of ethanolamine phosphatide[20]. The molar ratios given in Tables IV and V show further that in the cephalin fractions obtained from human grey matter and from rat and hen cerebrum about half the total phospholipid is present as plasmalogen, while in those from white matter, spinal cord and sciatic nerve 80–90 % of the phospholipid is in this form.

It was tentatively suggested some years ago by BRANTE[2], from calculations based partly on his own results and partly on earlier chemical and histological data on the distribution of lipids in the nervous system, that "most or all of the ethanolamine phospholipids contained in the myelin sheaths are present in the form of plasmalogens". The findings reported in this paper provide direct evidence in support of this suggestion.

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REFERENCES

[1] A. STAMMLER, U. STAMMLER AND H. DEBUCH, *Z. physiol. Chem., Hoppe Seyler's*, 296 (1954) 80.
[2] M. M. RAPPORT AND B. LERNER, *Biochim. Biophys. Acta*, 33 (1959) 319.
[3] H. CHRISTL, *Z. physiol. Chem., Hoppe Seyler's*, 293 (1953) 83.
[4] W. H. MINDER AND I. ABELIN, *Z. physiol. Chem., Hoppe Seyler's*, 298 (1954) 121.
[5] J. B. WITTENBERG, S. R. KOREY AND F. H. SWENSON, *J. Biol. Chem.*, 219 (1956) 39.
[6] J. FOLCH-PI, *Proc. 1st. Int. Neurochem. Symp.*, Acad. Press, N.Y., 1954, p. 121.
[7] S. R. KOREY AND M. ORCHEN, *Arch. Biochem. Biophys.*, 83 (1959) 381.
[8] A. N. DAVISON AND M. WAJDA, *J. Neurochem.*, 4 (1959) 353.
[9] N. E. ERICKSON AND W. E. M. LANDS, *Proc. Soc. Exptl. Biol.*, 102 (1959) 512.
[10] E. KLENK, H. DEBUCH AND H. DAUN, *Z. physiol. Chem., Hoppe Seyler's*, 292 (1953) 241.
[11] E. KLENK AND P. BÖHM, *Z. physiol. Chem., Hoppe Seyler's*, 288 (1951) 98.
[12] G. B. ANSELL AND J. M. NORMAN, *J. Neurochem.*, 1 (1956) 32.
[13] J. FOLCH, M. LEES AND G. H. SLOANE-STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
[14] D. N. RHODES AND C. H. LEA, *Biochem. J.*, 65 (1957) 526.
[15] L. ERNSTER, R. ZETTERSTRÖM AND O. LINDBERG, *Acta Chem. Scand.*, 4 (1950) 942.
[16] K. P. STRICKLAND, R. H. S. THOMPSON AND G. R. WEBSTER, *J. Neurol. Neurosurg. Psychiat.*, 19 (1956) 12.
[17] F. LEUPOLD AND H. BUTTNER, *Z. physiol. Chem., Hoppe Seyler's*, 292 (1953) 13.
[18] G. M. GRAY AND M. G. MACFARLANE, *Biochem. J.*, 70 (1958) 409.
[19] J. AXELROD, J. REICHENTHAL AND B. B. BRODIE, *J. Biol. Chem.*, 204 (1953) 903.
[20] J. FOLCH-PI AND F. N. LEBARON, in D. RICHTER, *Metabolism of the Nervous System*, Pergamon Press, London, 1957, p. 67.
[21] G. BRANTE, *Acta Physiol. Scand.*, vol. 18, (1949) Suppl. 63.

Biochim. Biophys. Acta, 44 (1960) 109–116

THE TRANSPORT OF PHOSPHATE IONS ACROSS THE HUMAN RED CELL MEMBRANE

I. THE DISTRIBUTION OF PHOSPHATE IONS IN EQUILIBRIUM AT COMPARATIVELY HIGH PHOSPHATE CONCENTRATIONS

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SUMMARY

1. The concentrations of chloride and phosphate ions in the cellular and extracellular phases have been determined in suspensions of human red cells incubated for about 3 h in salt solutions containing various concentrations of chloride and phosphate ions.

2. The concentration ratio of primary phosphate ions between the cellular and extracellular phases was in all cases found to be related to that of the chloride ions by a constant factor.

3. The significance of this result and its possible consequences for the problem of the mechanism of the transport of phosphate ions across the human erythrocyte membrane are discussed.

INTRODUCTION

To the understanding and elucidation of the mechanism of the phosphate ion penetration of the red cell membrane, a knowledge of the equilibrium concentrations of the phosphate ions on both sides of the membrane is essential, provided an equilibrium is established at all.

As early as in 1921, IVERSEN[1] tried to solve this problem by means of the very primitive methods of phosphate determination then available. Later determinations of the phosphate concentrations in serum and cells have been carried out by HALPERN on freshly drawn blood[2], and by MUELLER AND HASTINGS on incubated whole blood[3]. None of these works have, however, settled the problem quite satisfactorily. Often it has been taken for granted that if the distribution of phosphate ions between cells and serum were defined by passive diffusion processes, then such an equilibrium distribution would invariably exist in freshly drawn blood. Furthermore, such a passive distribution, which is found, *e.g.*, for chloride ions, has been assumed to be

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Abbreviations: ATP, adenosinetriphosphate; 2,3-DPGA, 2,3-diphosphoglyceric acid; TCA, trichloroacetic acid.

characterized by a constant concentration ratio cells/plasma. Consequently, it has not been deemed necessary to determine this ratio in each case.

During the last few years a great number of investigations on the $^{32}PO_4$ uptake of human erythrocytes have been performed. In some of these experiments optimal conditions were chosen for the uptake, and in others various enzyme poisons were added, notably fluoride. GOURLEY[4–6] and PRANKERD *et al.*[7], who have been particularly active in this field, have formulated a theory of the mechanism of uptake of phosphate ions in human erythrocytes. According to this theory the phosphate ions penetrate the membrane of the erythrocyte by an enzymic process in which extracellular inorganic phosphate is incorporated directly[5,6]—or through a few intermediate steps[7]—into the intracellular ATP or ATP located at the membrane.

This mechanism of penetration seems to make possible a thermodynamically active transport, as defined by ROSENBERG[8,9], so that Donnan distribution of the phosphate ions should be found only occasionally.

That the phosphate ions penetrate the red cell membrane or pores in it by diffusion has, however, been maintained by HAHN AND HEVESY[10] (working with rabbit red cells), by MUELLER AND HASTINGS[3], and by DUNKER AND PASSOW[11]. The findings of these authors seem to be irreconcilable with those of PRANKERD *et al.* and GOURLEY, although the last mentioned author will not exclude a small measure of diffusion in addition to the active uptake[5].

DUNKER AND PASSOW[11] have mainly been working with bovine and equine red cells. These cells are different from human red cells in several respects. In particular they have a very low glycolytic rate and a very low content of 2,3-DPGA[12], so conclusions regarding the mechanism of penetration into human red cells can hardly be drawn from experiments on bovine or equine red cells, or, for that matter, rabbit red cells.

In case the phosphate ions penetrate the membrane by diffusion, the membrane potential, which may be measured by the distribution of the chloride ions, determines the distribution of the phosphate ions between the cellular and extracellular phases.

In this paper are recorded our determinations of the equilibrium distribution of the monovalent phosphate ions at various concentrations, and since individual variations in pH, cell water content, and chloride ion distribution are recorded in the literature, we have measured these parameters in each experiment. Furthermore, we have endeavored to determine the distributions of phosphate and chloride ions under identical experimental conditions, in order to make errors originating in the determinations of cell volumes cancel out as far as possible.

METHODS

In the experiments, freshly drawn human blood was used throughout. The blood was taken from healthy adults and from patients of both sexes in the adult medical wards of the Finsen Memorial Hospital. These patients did not suffer from diseases affecting the red blood cells, except one with hypochromic anemia, (donor No. 10). Heparin was used as anticoagulant.

Immediately after being drawn, the blood was centrifuged at 1000 × *g* for 10 min, and the serum and the buffy coat were removed. The cells were washed twice with Ringer solution. About 20 ml of cells were suspended in an equal volume of a salt solution (see below) containing 200 mg glucose/100 ml. This suspension was incubated anaerobically in a 50-ml flask, which was gently rotated in a water bath for 2–5 h. The temperature of the bath was in all experiments 37.0 ± 0.2°. Generally the incubation was started 50–60 min after withdrawal of the blood.

The Ringer solution used for washing the cells had the following composition: 0.80 % NaCl, 0.037 % KCl, 0.014 % $CaCl_2$, 0.008 % $MgCl_2$, 0.01155 % KH_2PO_4, and 0.0634 % $Na_2HPO_4{,}2H_2O$, pH 7.4. The various salt solutions with different phosphate concentrations in which the cells were incubated, were prepared with the above solution as basis, given amounts of NaCl being replaced by $KH_2PO_4 + Na_2HPO_4$ so as to preserve a pH of 7.4 and isotonicity, and glucose being added. In all solutions for incubation, $CaCl_2$ was, however, omitted to prevent precipitation of calcium phosphate at higher phosphate concentrations.

After incubation, samples were taken from the suspension, and determinations of pH, chloride and inorganic phosphate concentrations, and dry weight were made. Another part of the suspension was centrifuged, and inorganic phosphate and chloride concentrations and dry weight were determined in cells and in extracellular phase.

Centrifugation was carried out in a centrifuge from Ole Dich, Denmark, holding twelve centrifuge tubes, each with a capacity of 400 μl. Suspensions were separated in the course of 45 sec at 13,000 × g. For the determination of chloride, inorganic phosphate, and dry weight, samples were taken with a 100 μl Carlsberg pipette from the supernatant and from the packed cell column. While the pipette used delivered 100.0 ± 1.0 μl of water, and presumably very closely the same volume of supernatant or TCA solution, it was found to deliver only 101.4 mg or 93.0 ± 1.5 μl of packed cells. Thus, the analytically determined quantities of chloride and phosphate ions refer to 100 μl of supernatant or to 93 μl of packed cells.

Trapped volume

The volume of extracellular phase between the cells in the packed column has been determined earlier with the aid of Evans Blue T 1824. The method is, as worked out by MAIZEL[13], a modification of a method used by SHOHL AND HUNTER[14] for determination of the percentage of serum in whole blood. In this determination even a slight degree of hemolysis will, however, cause serious disturbances in the colorimetric readings of the concentration of Evans Blue. We therefore chose to use the isotope ^{22}Na in the determination of the trapped volume in our experiments. The amount of ^{22}Na in the cell phase was determined 2–4 min and about 20 min after mixing the cells with a Ringer solution containing ^{22}Na. This procedure made it possible to correct for any uptake of ^{22}Na by the cells by extrapolation to zero time. However, this uptake proved to be of no significance at the prevailing temperature during the time intervals in question. As a mean of sixteen determinations we found the trapped volume to constitute 4.9 ± 0.2 μl or 5.2 ± 0.25 volume per cent of the cell phase samples. The experimental conditions in these determinations were in all respects identical with those of the normal experiments.

Chloride was determined mercurimetrically as follows: A quantity of 100 μl of extracellular phase or 93 μl of packed cells was mixed with 100 μl of 25 % (w/v) TCA by thorough stirring with a small glass rod. After 10 min the mixture was centrifuged for 2 min at 13000 × g, and 100 μl of supernatant was transferred to a tube containing 1 ml of water and 100 μl of 0.2 % diphenylcarbazone in 95 % ethanol.

The titration was performed with 0.002 *M* $Hg(NO_3)_2$ solution containing nitric acid in a concentration sufficient to prevent precipitation of basic salts. The amount of reagent consumed in a titration was 1–2 ml. Experiments showed that variations of 50 % in the TCA concentration had no influence on the color change or on the end-point of the titration. The standard error on the average of four samples was ± 0.46 % (70 quadruplicates).

pH values of the cell suspensions were always determined about 2 min after removal of the flask from the water bath. About 0.5 ml of the suspension was then transferred to a small glass vessel, and the pH value was read at once and after about 1 min. The temperature of the sample, measured with the aid of a thermocouple, was between 20 and 25°, the temperature of the room being 18 to 22°. To estimate the pH of the suspension at 37° the temperature coefficient of ROSENTHAL[15] was used:

$$\mathrm{pH}_{37} = \mathrm{pH}_t - 0.0147\,(37 - t)$$

where $t =$ the temperature at which the determination is made. The standard error can be estimated to be less than 0.05 pH unit. A semimicro glass electrode (Radiometer GA 222) and a semimicro calomel electrode (Radiometer K 401) were used.

Dry weight was determined in duplicate. Samples of about 100 µl were pipetted onto tared aluminium dishes and weighed. The samples were dried at 12–15 mm Hg and 60° for 16–20 h in a desiccator containing silica gel. This treatment proved to give constant weights. The relative standard error on the average of two determinations was ± 2.8 % (42 duplicates).

The volume of intracellular water, V_c, in the sample of packed cells was calculated from the percentage, t_c, (w/w), of cell dry matter as follows:

$$V_c = \left[101.4\left[\frac{100 - t_c}{100}\right] - 4.9\right]\,\mu\mathrm{l} \tag{1}$$

The first term in the right-hand member represents the total amount of water in the 93-µl sample (the density of the cell water being taken as one), while the second term represents the water content of the trapped extracellular phase. For the sake of convenience the above equation may be simplified to

$$V_c = (96.5 - 1.01\,t_c)\,\mu\mathrm{l} \tag{2}$$

Inorganic phosphorus

The procedure was developed on the basis of TAUSSKY AND SHORR's method[16]. The following molybdate stock solution was used: 50 g of ammonium molybdate is dissolved in about 400 ml 10 *N* sulfuric acid and diluted to 1000 ml with 10 *N* sulfuric acid. The reagent for analysis was prepared every day as follows: 10 ml stock solution was transferred to a 250-ml volumetric flask; about 150 ml water and 5 g $FeSO_4$, $7H_2O$ were added, and after dissolution the flask was filled up to its mark with water.

Procedure

A volume of 93 µl packed cells or 100 µl extracellular phase was added to 100 µl 25 % (w/v) TCA and thoroughly stirred with a small glass rod. After about 10 min the mixture was centrifuged for 2 min at 13,000 × *g*, and 100 µl supernatant was transferred to a tube containing 5 ml reagent. 15–20 min later the extinction was read at 740 mμ on a Beckman spectrophotometer, model DU. The phosphate content in μmoles was calculated from a standard curve, each value calculated as the average of four determinations. The relative standard error of this average was 0.48% (70 quadruplicates).

It is well known that this and similar methods always yield high values of the inorganic phosphate concentrations in solutions containing easily hydrolyzable organic phosphate. Since they are hydrolyzed, not only when protein is precipitated with TCA, but also in the strongly acid molybdenum reagent only insufficient protection of the organic phosphate is attained by precipitation at 0°.

LOWRY AND LOPEZ[17] and MARTIN AND DOTY[18] have developed methods which make it possible to determine inorganic phosphate in the presence of labile organic phosphates without causing the latter to hydrolyze. None of these methods could, however, be used directly to determine inorganic phosphate in samples of packed cells. When the method of LOWRY AND LOPEZ is applied to animal tissues, inhibition of the development of the molybdenum blue color often occurs[17]. This inhibition may be avoided by dilution, but in our case the dilution required would reduce the phosphate concentration to such a low value that it could not be determined with sufficient accuracy. If the method of MARTIN AND DOTY is applied directly, we find that a very stable emulsion is formed because of the high protein content of the erythrocytes. This emulsification takes place when the water phase containing molybdic and silicotungstic acids is extracted with an isobutanol–benzene mixture. While satisfactory separation is usually obtained within 30 sec, several minutes are required in the case of the erythrocytes.

In order to ascertain how much organic phosphate is hydrolyzed when we use our modification of the method of TAUSSKY AND SHORR, we modified the method of MARTIN AND DOTY and compared the results obtained with the two methods. Our modification is: One volume of cells is added to one volume of ice-cold TCA and stirred thoroughly. After storage for 10 min in an ice bath, the mixture is centrifuged at 0°, and 100 or 200 μl of supernatant is treated according to MARTIN AND DOTY, except that silicotungstic acid is omitted. The volumes were changed to give greater sensitivity. By means of ^{32}P phosphate we examined the efficiency of the extraction of the phosphomolybdic acid under these somewhat changed conditions; we found that it was still excellent with fifteen seconds' shaking.

This modification of the method of MARTIN AND DOTY could not be used as a routine procedure for technical reasons, but it was useful for checking the method generally used. Erythrocytes from freshly drawn blood were washed with isotonic sodium chloride solution, and phosphate was determined by both methods. A series of comparative experiments showed that, on an average of six determinations, 0.30 μg P ± 30% was split/93-μl sample of cells in our routine method, the values obtained with the modified method of MARTIN AND DOTY being taken as true. The lowest intracellular level of inorganic phosphate we found in this series of experiments was 0.272 μmoles/93 μl packed cells. Hence the numerically greatest correction for hydrolyzed organic phosphate was — 3.7 ± 1.1%.

The recovery factor

As stated above, the concentration of chloride and phosphate ions in the intracellular phase were determined in the following way: A volume of 93 μl of packed cells was transferred by means of a Carlsberg pipette to a micro centrifuge tube containing 100 μl 25 % (w/v) TCA. After stirring, standing, and collection of the protein trichloroacetate precipitate by centrifugation, 100 μl of the clear supernatant was withdrawn with a Carlsberg pipette pending, further analysis.

When the data obtained with the clear supernatant are to be interpreted, a number of phenomena must be taken into consideration. In the first place, mixing of 93 μl of cells with 100 μl of 25 % TCA will lead to a volume change so that the volume after mixing will not be 193 μl. In the second place, the protein trichloracetate precipitate will occupy a considerable proportion (about 20 %) of the total volume, so the volume available to the chloride and phosphate ions is considerably smaller than the total volume. Finally, the precipitate may bind greater or smaller amounts of anions and cations by occlusion and adsorption. The amounts of the individual ions thus bound may depend, *inter alia*, on the signs and magnitudes of their charges.

The proportions of the quantities of chloride and phosphate ions present in the intracellular phase that are removed with the 100 μl of supernatant must therefore be determined experimentally for each individual ion. We attempted in a special series of experiments to determine this recovery factor for chloride and for phosphate ions. This was done in the way that the data obtained for each individual blood sample were related to the result of a dry matter determination carried out at the same time.

Outline of the method

Volumes of 93 μl of packed cells and 100 μl of 25 % (w/v) TCA to which sodium chloride or [^{32}P]phosphate had been added (TCA*), were mixed with vigorous stirring, allowed to stand for 10 min, and centrifuged for 2 min at 13000 × *g*. Then the relative contents of chloride ions and of ^{32}P in 100 μl TCA* and in 100 μl supernatant were determined. Chloride was determined by the mercurimetric method described and ^{32}P was determined by measurement of bremsstrahlung in a well-type scintillation crystal. In the case of the chloride ions it was necessary to carry out a blank determination to make it possible to introduce a correction for the quantity of chloride ion already present in the cells.

As a result of this investigation it was found that the recovery factor, R_F, was, in terms of per cent, $(1.75 \pm 0.02)\ t_c$ (25 determinations). This was true of phosphate as well as of chloride ions. Thus, the quantity of, *e.g.*, chloride ions in a sample of packed cells is given by

$$M = \frac{100\ \text{Cl}_c}{1.75\ t_c}\ \mu\text{moles} \tag{3}$$

where Cl_c is the number of μmoles determined in 100 μl supernatant by the mercurimetric titration. For determination of extracellular concentrations, the recovery percentage was found to be exactly 50.0 for phosphate as well as chloride ions.

RESULTS

Table I presents the results of thirteen experiments with blood from ten donors. Not included are several preliminary, less accurate experiments, which have, however, given very similar results. The concentrations of chloride and phosphate ions in the

TABLE I

RESULTS OF THIRTEEN EXPERIMENTS WITH BLOOD FROM TEN DONORS

P_t = total inorganic phosphate, ex = extracellular phase, c = cellular phase, $R_{c_2}\gamma_{c_2}$ and $R_{P^-}\gamma_{P^-}$ are defined in eqns. 6 and 14, $Q = R_{P^-}\gamma_{P^-}/R_{c_2}\gamma_{c_2}$.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Expt.	Donor No.	Incubation medium Concn. in mmol/l of:		µmole phosphate in 100 µl supernatant from precipitation of:		µmole chloride in 100 µl supernatant from precipitation of:		Time of incubation in min.	pH corr. to 37°	Dry weight per cent				Average of Q for each conc. interval
		Cl	P_t	ex	c	ex	c			t_c	$R_{Cl}\gamma_{Cl}$	$R_{p-}\gamma_{p-}$	Q	
1	1	95.1	40.0	1.44	0.778	5.72	3.68	170	7.11	34.4	0.785	0.752	0.96	
2	2	95.1	40.0	1.27	0.663	5.52	3.52	185	7.08	35.7	0.764	0.721	0.94	
3	3	95.1	40.0	1.45	0.791	5.72	3.72	215	7.05	35.8	0.780	0.744	0.95	0.98
4	4	95.1	40.0	1.29	0.627	6.96	4.08	150	7.19	38.4	0.671	0.709	1.06	
5	5	95.1	40.0	1.30	0.766	5.60	3.80	285	7.12	35.2	0.824	0.795	0.97	
6	6	119.3	22.2	0.769	0.442	6.32	4.04	200	7.00	34.7	0.776	0.798	1.01	
7	7	119.3	22.2	0.729	0.392	6.48	4.16	240	7.13	35.9	0.767	0.756	0.97	0.99
8	8	119.3	22.2	0.800	0.466	6.48	4.20	240	7.13	34.3	0.793	0.816	1.01	
9	9	119.3	22.2	0.785	0.465	6.52	4.44	255	7.09	35.2	0.827	0.795	0.95	
10	7	131.5	13.3	0.483	0.262	6.92	4.44	190	7.19	35.0	0.777	0.762	0.96	
11	10	131.5	13.3	0.520	0.356	7.08	5.08	210	6.98	29.9	0.960	0.924	0.95	1.00
12	8	131.5	13.3	0.505	0.277	7.00	4.40	195	7.18	33.5	0.777	0.789	1.00	
13	9	131.5	13.3	0.515	0.305	7.24	4.64	220	7.12	35.3	0.773	0.834	1.06	

salt solution in which the twice washed cells are incubated are stated in Columns 2 and 3. Attention is called to the fact that before incubation the cells are always washed with the salt solution mentioned under METHODS. In the following columns are listed the final quantities of phosphate and chloride ions found in 100 μl supernatant from precipitated extra- and intracellular samples, the time of incubation being given in Column 8. The phosphate quantities from the samples of intracellular phase have been corrected for organic phosphate hydrolyzed, as discussed under METHODS.

The absence of any correlation between the values of Q (see page 19) and the corresponding incubation times as well as experience from [^{32}P]phosphate exchange experiments have convinced us that incubation periods of about 3 h are sufficient for establishment of equilibrium. This is in accord with the result of IVERSEN[1], who added isotonic Na_2HPO_4 solution to defibrinated blood (rabbit blood, to be sure). His data show that the added phosphate distributed itself uniformly in the course of 2–4 h.

The percentage of dry matter in the packed cells is listed in Column 10. As a mean of all determinations (except the one on a patient with hypochromic anemia, donor No. 10), we have found this percentage to be 35.3 % ± 1 % relative. However, it must be borne in mind that the experimentally determined values for the cell dry matter ought to be corrected for the volume of trapped extracellular phase for calculation of the percentage of dry matter in the cells themselves.

In their work on the distribution of ^{32}P-labelled phosphate ions between cells and serum in human blood, EISENMAN, OTT, SMITH AND WINKLER[19] used a value for cell dry matter of 28 %. This value, the mean of ninety-four analyses, was taken from the work of EISENMAN, MACKENZIE AND PETERS[20]. Here, however, the percentage of dry matter in the cells was computed from measurements of dry matter in serum and whole blood and of hematocrit. The very low figures may thus be explained by a high percentage of trapped plasma in the packed cell column of the hematocrit capillary. In addition to the results listed in the table the following values have been determined in each single experiment: the percentage of dry matter in the extracellular phase and the percentages of dry matter and of cell volume in the cell suspension. In the thirteen experiments, the dry matter in the extracellular phase has varied between 1.2 and 1.8 % (w/w), most of which, about 1 %, has been inorganic salts. Consequently, no correction for extracellular dry matter has been introduced. This result further shows that if any hemolysis has taken place, it must have been very slight. For the cell suspension as a whole, the content of dry matter has been 16.0 ± 1.4 % (w/w) (not including donor No. 10, the one with hypochromic anemia). This result as well as the cell volume of 41.6 ± 1.9 % may be considered an expression of the uniformity of the suspensions incubated. The cell volumes have been determined in quadruplicate by centrifugation in short capillary tubes for 2 min at 13,000 × g. As will be seen from the pH values listed in Column 9 of the table, the pH has fallen during the incubation to a value 0.2 to 0.4 unit lower than the initial value, pH 7.4, of the salt solution incubated.

Calculations

In the interpretation of these analytical data we have made the assumption that all the water inside the cells is free and osmotically available so that the total volume,

V_c, should be used in the calculation of the thermodynamic activities of the dissolved ions. HUTCHINSON[21] has found in experiments with ^{14}C-tagged alcohols and $H_2{}^{18}O$ that these substances seem to mix into all the water of human erythrocytes, indicating that as far as these substances are concerned practically all the water inside the cell is free.

On the basis of the assumption that all the intracellular water is available, the ratio of the intracellular to the extracellular chloride ion activity, $R_{Cl} = {}^aCl_c/{}^aCl_{ex}$, is calculated in the following manner. Since the R_F for determination of extracellular matter is 50 %, the concentration of chloride ion in the extracellular phase is given by eqn. 4.

$$[Cl_{ex}] = \frac{2\ Cl_{ex}}{100} \text{ moles/l} \tag{4}$$

where Cl_{ex} is the number of μmoles of chloride ion/100 μl of supernatant as determined by the mercurimetric titration. The chloride concentration in the intracellular phase is obtained from eqn. 5:

$$[Cl_c] = \frac{\left(\dfrac{100\ Cl_c}{1.75\ t_c} - 4.9 \cdot \dfrac{2\ Cl_{ex}}{100}\right)}{96.5 - 1.01\ t_c} \text{ moles/l cell water} \tag{5}$$

In this expression, the first term of the numerator represents the total quantity of chloride ions, in terms of μmoles, contained in the 93-μl sample of packed cells; *cf.* eqn. 3. The second term of the numerator represents the amount of chloride ion contained in the 4.9 μl trapped extracellular phase. Finally, the denominator is the expression of eqn. 2 for the volume of the intracellular aqueous phase contained in the 93 μl packed cells.

Now R_{Cl} is found by division of eqns. 4 and 5:

$$R_{Cl}\gamma_{Cl} = \frac{[Cl_c]}{[Cl_{ex}]} = \frac{\left(\dfrac{100\ Cl_c}{1.75\ t_c} - 0.098\ Cl_{ex}\right) 50}{96.5 - 1.01\ t_c)\ Cl_{ex}} \tag{6}$$

where γ_{Cl} is the ratio of the activity coefficients of the chloride ions in the extra- and intracellular phases, respectively (${}^fCl_{ex}/{}^fCl_c$).

The distribution ratio, R_{Cl}, determined in each individual experiment by means of eqn. 6, is used as an expression of the Donnan distribution in the given cell suspension. Consequently, this is the magnitude with which the distribution ratio calculated for the monovalent phosphate ions is to be compared.

In the course of the derivation of the equation for the distribution ratio $R_{P^-} = {}^aP^-{}_c/{}^aP^-{}_{ex}$ (the subscript $_{P^-}$ referring to the monovalent phosphate ion $H_2PO_4^-$), we first arrive at an equation analogous to (6), which gives us, however, the concentration ratio of total inorganic intracellular to extracellular phosphate. The concentrations of the monovalent phosphate ions are calculated from the concentrations of total inorganic phosphate by means of eqn. 9, which is obtained by combination of eqns. 7 and 8:

$$K'' = \frac{[HPO_4{}^{--}]\ {}^aH^+}{[H_2PO_4{}^-]} \tag{7}$$

$$[H_3PO_4] + [H_2PO_4{}^-] + [HPO_4{}^{--}] + [PO_4{}^{---}] = [P_{total}] \tag{8}$$

At pH values between 7.0 and 7.2, the concentrations of unionized and completely ionized phosphoric acid are negligible. When they are left out, combination of eqns. 7 and 8 gives 9:

$$[H_2PO_4^-] = \frac{{}^aH^+}{({}^aH^+ + K'')}[P_{total}] \tag{9}$$

This equation is valid for calculation of the concentration of the monovalent phosphate ions in the extracellular as well as the intracellular phase. In eqn. 9 the hydrogen ion activity in the extracellular phase is the measured value in the cell suspension corrected to 37°. The corresponding value in the cellular phase, ${}^aH^+{}_c$, is calculated by means of eqn. 10 and the resulting 11:

$$R_{Cl} = \frac{{}^aCl_c^-}{{}^aCl_{ex}^-} = \frac{{}^aOH_c^-}{{}^aOH_{ex}^-} = \frac{{}^aH_{ex}^+}{{}^aH_c^+} \tag{10}$$

$${}^aH_c^+ = \frac{{}^aH_{ex}^+}{R_{Cl}} \tag{11}$$

This presupposes that the hydroxyl ions (partly by a rapid equilibration of the HCO_3^--ions) are distributed among the intra- and extracellular phases in the same concentration ratio as the chloride ions. As for R_{Cl} we are unable to calculate an accurate value of it. Experimentally we determine the concentration ratio $[Cl_c]/[Cl_{ex}] = R_{Cl}\gamma_{Cl}$. Considering the uncertainty of the above estimation of intracellular ionic strength, we have not tried to calculate γ_{Cl}, but have simply taken it as unity.

Let P_c^t and P_{ex}^t denote the analytically determined concentrations of inorganic phosphate in the intra- and extracellular phases, respectively. The corresponding concentrations of monovalent phosphate ions may then be calculated, partly from a combination of eqns. 9 and 11, and partly from eqn. 9:

$$[P_c^-] = [P_c^t]\frac{{}^aH_{ex}^+}{(K''_c R_{Cl} + {}^aH_{ex}^+)} \tag{12}$$

$$[P_{ex}^-] = [P_{ex}^t]\frac{{}^aH_{ex}^+}{(K''_{ex} + {}^aH_{ex}^+)} \tag{9a}$$

From eqns. 9a and 12 we obtain eqn. 13:

$$R_{P^-} = \frac{{}^aP_c^-}{{}^aP_{ex}^-} = \frac{1}{\gamma_{P^-}}\frac{[P_c^t]}{[P_{ex}^t]}\frac{(K''_{ex} + {}^aH_{ex}^+)}{(K''_c R_{Cl} + {}^aH_{ex}^+)} \tag{13}$$

This shows that the equation giving the distribution ratio $R_{P^-}\gamma_{P^-}$ may be obtained from an equation analogous to 6 by multiplying its right-hand member by the last fraction occurring in 13:

$$R_{P^-}\gamma_{P^-} = \frac{\left(\frac{100}{1.75\, t_c} P_c - 0.098\, P_{ex}\right) 50\, (K''_{ex} + {}^aH_{ex}^+)}{(96.5 - 1.01\, t_c)\, P_{ex} \quad (K''_c R_{Cl} + {}^aH_{ex}^+)} \tag{14}$$

In addition to the experimentally determined phosphate quantities, this equation contains the values K''_{ex}, K''_{c}, R_{Cl} and $\gamma_{\mathrm{P^-}} = f_{\mathrm{P^-ex}}/f_{\mathrm{P^-c}}$. The second ionization constant of phosphoric acid (eqn. 7) was computed from the equation given by BJERRUM[22] for the ionic strength range up to 0.1:

$$\mathrm{p}K'' = 7.165 - 1.545\sqrt{\mu} + 1.12\,\mu \qquad (15)$$

where μ is the ionic strength of the solution. The ionic strength of the extracellular phase, μ_{ex}, can easily be calculated with sufficient accuracy, and we have found it to vary between 0.160 and 0.185. In expts. 1 through 5 we have chosen to use the value 0.18 and in expts. 6 through 13 the value 0.17, which give $\mathrm{p}K''_{\mathrm{ex}}$ values of 6.71 and 6.72, respectively. The ionic strength in the intracellular phase cannot be computed, but considering the content of organic phosphates in this phase we have chosen to use the value 0.20, giving a $\mathrm{p}K''_{\mathrm{c}}$ value of 6.70.

We have chosen to express the final result of an experiment by the ratio $Q = R_{\mathrm{P^-}}\gamma_{\mathrm{P^-}}/R_{\mathrm{Cl}}\gamma_{\mathrm{Cl}}$, and have not attempted to calculate the activity coefficients of the monovalent phosphate and chloride ions in the extra- and intracellular phases. If, however, the ratios of the activity coefficients in the extra- to the intracellular phases are identical for primary phosphate and for chloride ions, Q should be close to unity provided that the distribution of the primary phosphate ions is determined solely by the Donnan potential, and provided, of course, that all the constants used have been chosen correctly.

As a mean of twelve determinations (not including donor No. 10, the one with hypochromic anemia), $R_{\mathrm{Cl}}\gamma_{\mathrm{Cl}}$ was found to be 0.78 ± 0.01. The average value of Q found from all thirteen experiments was 0.98 ± 0.01, the relative standard deviation of a single determination of Q being 4.2 %.

The standard deviation to be expected for a single Q value was calculated in the following manner: The partial derivatives of Q with respect to the various parameters involved were calculated by means of the equation for Q obtained by division of eqn. 14 by eqn. 6. In the calculation were used the average values pH = 7.1, $R_{\mathrm{Cl}}\gamma_{\mathrm{Cl}} = 0.78$, $R_{\mathrm{P}}t = 0.68$, and $t_{\mathrm{c}} = 35$, and for $\mathrm{p}K''_{\mathrm{ex}}$ and $\mathrm{p}K''_{\mathrm{c}}$ the value 6.70. For the standard deviations of the values for chloride, phosphate, dry matter, and pH, the figures quoted under METHODS were used. For $\mathrm{p}K''_{\mathrm{ex}}$ and $\mathrm{p}K''_{\mathrm{c}}$ the standard deviations were taken as 0.005 and 0.01, respectively (see below). In this way the relative standard deviation for a single Q determination was calculated as 2.5 %, a somewhat lower value than was found experimentally.

Column 14 of the table shows that Q seems to rise slightly when the equilibrium concentration of phosphate decreases. This apparent rise, which amounts to 2 % within our concentration interval, might be due to a too small correction for organic phosphate hydrolyzed, or the $R_{\mathrm{P^-}}\gamma_{\mathrm{P^-}}$ value might be subject to a systematic error due to possible adsorption of phosphate ions to the membrane or stroma, since the expression for $R_{\mathrm{P^-}}\gamma_{\mathrm{P^-}}$ was derived on the assumption that the concentration of free phosphate ions was equal to the analytically determined phosphate concentration. It is difficult to make an estimate of the magnitude of an error of this kind, but the suggested correlation between Q and the found phosphate concentration may be explained by the occurrence of such an adsorption. However, experiments with phosphate labelled with ^{32}P have given results indicating only a very slight effect of adsorption.

Among the systematic sources of error the choises of the values of R_F and pK''_c are particularly important. In the section on methods, R_F has been given as $(1.75 \pm 0.02)\ t_c$. The standard deviation of 0.02 stems partly from the uncertainty of the analytical determination, partly from a possible variation in R_F from one precipitation to another, which is, however, involved in the quadruplicate determinations of the phosphate and chloride concentrations. A relative change of 1% in R_F gives rise to a relative change in Q of 0.8%. The pK'' depends on the ionic strength, which was easily calculated for the extracellular phase with reasonable accuracy. In the numerical treatment of the experimental data we even used two different values of pK''_{ex}, so we feel justified in ascribing a small standard deviation to this magnitude. As already mentioned, however, it is very difficult to motivate the choice of any value of ionic strength of the intracellular phase. According to eqn. 15, a change of μ_c from for example 0.18 to 0.20 changes the value of pK''_c from 6.71 to 6.70, corresponding to a change of −1.6% in Q.

Finally it should be mentioned that in expt. 4, Q was determined not only at $t = 150$ min, but also after 20 and 40 min. The results were: at $t = 20$ min, $Q = 0.55$, at $t = 40$ min, $Q = 0.85$; and at $t = 150$ min, $Q = 1.06$.

DISCUSSION

The results and considerations reported in the foregoing sections suggest that, under the given experimental conditions, the distribution of inorganic phosphate ions between the intra- and the extracellular phases is governed by a simple principle. In this connection the absolute value of Q is less significant than the rather pronounced constancy of the Q value found within the rather large concentration interval studied. In our opinion, such a regular distribution can only be found if the phosphate ions pass the erythrocyte membrane chiefly by a passive penetration process.

This passive process might consist in a simple diffusion of free phosphate ions through the membrane or pores in it. Another possibility would be that a complex of phosphate ion with a carrier molecule was formed at the surface of the membrane, diffused through, and decomposed at the other side of the membrane. Such a passive carrier transport mechanism of penetration of the human erythrocyte membrane by glucose has been demonstrated by ROSENBERG AND WILBRANDT[23, 24].

Our finding is at variance with the conclusions from studies carried out by HALPERN[2], by EISENMAN, OTT, SMITH AND WINKLER[19], and with the results of MUELLER AND HASTINGS[3]. HALPERN[2] and EISENMAN *et al.*[19] find the distribution of phosphate ions in human whole blood (freshly drawn, respectively incubated) so irregular that an active uptake of phosphate ions seems to them the only reasonable assumption. HALPERN[2] even claims to have observed active transport of phosphate ions in both directions through the membrane. Also MUELLER AND HASTINGS[3] find a definitely irregular distribution, but most often with considerably higher intracellular phosphate ion concentrations than the values calculated, according to the Donnan distribution, from the concentrations in the corresponding sera. Nevertheless, these authors conclude, partly on other grounds, that the membrane is penetrated by slow diffusion.

These earlier investigations were done on freshly drawn or incubated whole blood. In contrast, all of our studies were performed with washed cells that had been incubated for shorter or longer times in salt solutions containing glucose. Furthermore, it is undoubtedly an important fact that our experiments were carried out with total inorganic phosphate concentrations considerably higher than in the experiments of the above authors. In this connection, the significance of the large pool of organic phosphate in the human erythrocyte should be strongly emphasized. The size of this pool, of which about 40% is 2,3-DPGA[25], may fluctuate considerably because of phosphorylation and dephosphorylation, *in vitro* as well as *in vivo*[25]. In work with phosphate ions a suspension of human erythrocytes will thus behave like a polycompartment system containing extracellular inorganic phosphate, intracellular inorganic phosphate, and intracellular organic phosphate. Since the phosphate ion penetrates the erythrocyte membrane rather slowly, decomposition of a relatively small amount of organic phosphate will be able to give rise to a considerable concentration gradient across the membrane if the total inorganic phosphate concentration is as low as 1 or 2 mmoles/l suspension or whole blood. In such a case considerable fluctuations around the concentration ratio corresponding to passive distribution might be expected. In incubation of whole blood a certain dephosphorylation often takes place, and consequently increased amounts of intracellular inorganic phosphate are often found in such incubation experiments. If, however, the total inorganic phosphate concentration is somewhat higher, say 5 to 6 mmoles/l, this effect of the organic phosphate pool hardly makes itself felt.

As mentioned before, most of the available analytical methods for determination of inorganic phosphate in the cell phase lead to some hydrolysis of organic phosphate. In none of the works mentioned has this hydrolysis been adequately controlled, and so greater or smaller quantities of the organic phosphate present have presumably been determined as inorganic phosphate. Therefore, the most favourable conditions for a correct determination of the inorganic phosphate in the cell phase are those of comparatively high concentrations of inorganic phosphate, relative to which the percentage of organic phosphate erroneously included will be at a minimum.

The results given here do not, however, exclude the occurrence *in vivo* of some active transport of the phosphate ions, a transport which might make itself felt at the low phosphate concentrations prevailing in the plasma.

REFERENCES

[1] P. Iversen, *Biochem. Z.*, 114 (1921) 297.
[2] L. Halpern, *J. Biol. Chem.*, 114 (1936) 747.
[3] C. B. Mueller and A. B. Hastings, *J. Biol. Chem.*, 189 (1951) 869.
[4] D. R. H. Gourley, *Am. J. Physiol.*, 164 (1951) 213.
[5] D. R. H. Gourley, *Arch. Biochem. Biophys.*, 40 (1952) 1.
[6] D. R. H. Gourley, *Virginia J. Sci.*, 5 (1954) 20.
[7] T. A. J. Prankerd and K. I. Altman, *Biochem. J.*, 58 (1954) 622.
[8] Th. Rosenberg, *Acta Chem. Scand.*, 2 (1948) 14.
[9] Th. Rosenberg, *Symposia Soc. Exptl. Biol.*, 8 (1954) 27.
[10] L. Hahn and G. Hevesy, *Acta Physiol. Scand.*, 3 (1941–42) 193.
[11] E. Dunker and H. Passow, *Pflügers Archiv.*, 256 (1953) 446.
[12] S. Rapoport and G. M. Guest, *J. Biol. Chem.*, 138 (1941) 269.
[13] M. Maizels, *Quart. J. Exptl. Physiol.*, 33 (1946) 129.
[14] A. T. Shohl and T. H. Hunter, *J. Lab. Clin. Med.*, 26 (1941) 1829.
[15] T. B. Rosenthal, *J. Biol. Chem.*, 173 (1948) 25.
[16] H. H. Taussky and E. Shorr, *J. Biol. Chem.*, 202 (1953) 675.
[17] O. H. Lowry and J. A. Lopez, *J. Biol. Chem.*, 162 (1946) 421.

[18] J. B. Martin and D. M. Doty, *Anal. Chem.*, 21 (1949) 965.
[19] A. J. Eisenman, L. Ott, P. K. Smith and A. W. Winkler, *J. Biol. Chem.*, 135 (1940) 165.
[20] A. J. Eisenman, L. B. Mackenzie and J. P. Peters, *J. Biol. Chem.*, 116 (1936) 33.
[21] E. Hutchinson, *Arch. Biochem. Biophys.*, 38 (1952) 35.
[22] N. Bjerrum and A. Unmack, *Kgl. Danske Videnskab. Selskab, Mat. fys. Medd.*, 9 (1929).
[23] Th. Rosenberg and W. Wilbrandt, *J. Gen. Physiol.*, 41 (1957) 289.
[24] W. Wilbrandt, S. Frei and Th. Rosenberg, *Exptl. Cell. Research*, 11 (1956) 59.
[25] S. Rapoport and G. M. Guest, *J. Biol. Chem.*, 138 (1941) 269.
[26] M. Rohdewald and M. Weber, *Z. physiol. Chem., Hoppe-Seyler's*, 306 (1956) 90.

THE HAEMOLYSIS OF HUMAN ERYTHROCYTES IN RELATION TO THE LATTICE STRUCTURE OF WATER*

I. DELAYED HAEMOLYSIS IN HYPOTONIC MALONAMIDE SOLUTIONS

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SUMMARY

The haemolysis of human erythrocytes in hypotonic aqueous malonamide has been examined in relation to the quasi-crystalline properties of liquid water. It is suggested that the controlling factor in the mechanism of this process is the order promoting effect of malonamide on the water lattice; this suggestion is supported by a study of the kinetics of malonamide induced haemolysis.

INTRODUCTION

During an investigation of the permeability of human erythrocytes to organic nitrogenous substances, in which the haemolysis technique was employed, a delayed action effect was observed with hypotonic solutions of malonamide.

A similar effect occurs with hypotonic solutions of glucose and this has been described by HENDRY[1] as delayed haemolysis.

The results of an investigation of delayed haemolysis in hypotonic malonamide solutions are described.

MATERIALS AND METHODS

Preparation of the blood

The experiments reported here were carried out on fresh, normal human blood. Immediately after its withdrawal by venepuncture, a 20-ml sample of blood was

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discharged from the syringe into a clean, dry test tube containing 2 mg heparin powder; the tube was stoppered and the contents thoroughly mixed by repeated gentle inversion. After mixing, the blood was transferred to a tonometer and agitated gently for at least 20 min, at room temperature and pressure, in an atmosphere of moist oxygen. The blood received no treatment other than oxygenation and remained in the tonometer until required.

Preparation of malonamide solutions

The malonamide was purified by boiling with active charcoal and recrystallising twice from acetone. The purified material was dried *in vacuo* and stored over conc. sulphuric acid. Fresh solutions were prepared daily.

Since the erythrocyte is highly osmosensitive the potential osmotic pressure of the suspending medium is a major factor in this work. Accordingly osmotic pressures were derived from freezing point depression data by the equation[2]

$$\pi = -\frac{LT}{V_1 T_0^2} \Delta T$$

L denotes the molar latent heat of fusion of the solvent, $\overline{V}_1$ is the partial molar volume, and is, for dilute solutions, equivalent to V', the molar volume of the solvent; T is the absolute temperature of the solution, T_0 the freezing point of the pure solvent and ΔT the freezing point depression due to added solute.

The equation simplifies to,

$$\pi_t = (-12.02)\frac{273 + t}{273}(-\Delta t)$$

where π_t is the osmotic pressure, in atmospheres at t^0, of a dilute aqueous solution having the freezing point depression Δt.

TABLE I

OSMOTIC PRESSURES OF MALONAMIDE SOLUTIONS

Osmotic pressure (Atm)	*Temperature °C*	*Malonamide (g/100 ml)*
3.5	10	1.539
	20	1.489
	30	1.439
5.0	10	2.196
	20	2.125
	30	2.056
6.5	10	2.855
	20	2.761
	30	2.670

The osmotic pressure–solute concentration–temperature relationships for some of the malonamide solutions used are illustrated in Table I.

The use of this equation is advocated because it provides a common denominator for expressing an important physical property of solutions, irrespective of the solute species.

The haemolysing system

A standard dilution of 1 part by volume whole blood to 20 parts by volume solution was used throughout this work. With normal blood therefore, of packed cell volume 45 %, the plasma is diluted 1:38 and the osmotic contribution of the plasma solutes is about 0.2 atm.; the effect of temperature change on this value is small enough to be neglected. It should be remembered, however, that although the osmotic pressure of the solution is quoted in the following experiments, the osmotic pressure of the haemolysing system is greater by 0.2 atm.

In aqueous solution malonamide has a weakly acid reaction and this, together with dissolved carbon dioxide, gives a pH range of 4.0–5.5 at the concentrations used. The buffering power of the plasma is sufficient to bring the pH of the system up to the alkaline side of neutrality. In the experiments reported here the pH was checked at intervals and found to be within the range 7.2–7.7 units.

Experimental techniques

At the beginning of each experiment the malonamide solution, contained in a large test tube with ground glass stopper, was placed in a thermostatic water bath to attain working temperature. Fully oxygenated whole blood in the required proportion, was then added by pipette; the system was thoroughly mixed and the tube returned to the water bath.

At suitable intervals of time, 3-ml sampleswere with drawn and the intact cells centrifuged down at 4000 rev./min for 30 sec in an MSE Minor centrifuge. After centrifugation a 2.0-ml sample of the supernatant was withdrawn by pipette and its haemoglobin content measured by the alkaline haematin method of CLEGG AND KING[4]; colour intensity was measured in the Spekker photoelectric absorptiometer, using a 1.00-cm cell and No. 604 spectrum green filters. At the end of the experiment an uncentrifuged 2.0-ml sample was treated in the same way, giving a value for complete haemolysis; the degree of haemolysis in each of the partially haemolysed samples was expressed as a percentage of this figure. The rate curves were obtained by plotting percent haemolysis to a base of time.

The experimental rate was taken as the average slope of the haemolysis curve between 25 % and 75 % haemolysis. The time factor is very important, so a stop clock was employed and a standard procedure for the withdrawal and centrifugation of samples was established and adhered to throughout the work.

For the determination of rates in rapidly haemolysing systems the following procedure was adopted. Using a 1.00-ml graduated pipette. 0.15 ml oxygenated whole blood was added to 3.0 ml distilled water in a small test tube carried in a rack in the thermostatic water bath; the instant the addition of blood was complete, the tube was removed from the bath, shaken twice and returned. The end point, complete haemolysis, was obtained by periodically removing the tube from the water bath and holding it against a printed page in strong light; at 100 % haemolysis, when the system becomes quite clear, the watch was stopped. The rate was expressed as percent haemolysis per second. This method, of course, can only be employed when haemolysis is rapid and complete.

RESULTS

Haemolysis in hypotonic malonamide solutions at constant temperature

When fully oxygenated whole blood is diluted in the ratio 1:21 with slightly hypotonic malonamide solution (6.5 atm. at 20°) 55–60 min elapse before the erythrocytes are completely haemolysed. Of this period there is a zero haemolysis lag phase lasting 15 min, after which haemolysis begins and accelerates into the main lytic phase of about 20 min duration; the rate then decreases as the system approaches complete haemolysis. The graph of the process (Fig. 1) is a sigmoid curve.

In a solution of 5.0 atm. at 20° the lag phase is reduced to 10 min; the main lytic phase occupies about 10 min and haemolysis is complete in 35 min. At 3.5 atm the lag phase is reduced to around 5 min and the process is complete in 21 min. When the osmotic pressure of the solution is 2.0 atm there is no lag phase, about 50 % haemolysis occurs within 1 min of adding the blood and the remaining cells are completely haemolysed within 5 min.

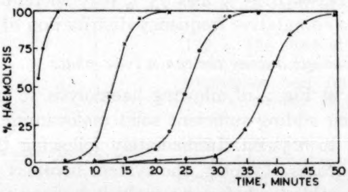

Fig. 1. The haemolysis of human erythrocytes in hypotonic malonamide solutions at 20°. ●—●, 2.0 atm.; ×—×, 3.5 atm.; ○—○, 5.0 atm.; ▲—▲, 6.5 atm.

Immediately after mixing blood and solution, in the range 3.5–6.5 atm, the system is opaque to the naked eye and has the characteristic sheen of a discoid cell suspension. On standing the cells swell, the system becomes translucent and soon afterwards a trace of haemolysis is detectable.

All existing evidence shows that osmotic swelling, which is governed by the relative intra- and extracellular osmotic pressures, is complete within a matter of seconds; it is the result of the movement of water, to which the membrane offers no apparent resistance. This accounts for the initial 50 % haemolysis observed in the system of lowest osmotic pressure; it accounts also for the decrease in the duration of the lag phase with decreasing osmotic pressure, since the lower the osmotic pressure of the solution, the greater the initial rapid swelling.

The occurrence of delayed haemolysis in aqueous malonamide, irrespective of the degree of hypotonicity, shows that swelling continues beyond the point necessary for the restoration of simple osmotic equilibrium. This continued swelling is due to displacement of the rapidly established osmotic equilibrium by the penetration of malonamide, which diffuses into the cell in the direction of its concentration gradient. The increase in intracellular malonamide necessitates a corresponding increase in intracellular water to maintain osmotic equilibrium, the excess water causes further swelling and the process continues until haemolysis occurs.

In the main lytic phase the rate of haemolysis increases as the osmotic pressure decreases. The reason for this is not immediately obvious since the cells become prolytic spheres, irrespective of the tonicity of the medium, before this stage of haemolysis is reached. Haemolysis is the result of stresses imposed by the accumulation of excess water within the cell and this accumulation of excess water is induced by the penetration of malonamide; the rate of haemolysis therefore will depend on the conditions which control one or other, or both of these factors. The experiments indicate that the volume of water required to haemolyse the cell is less in more dilute solutions and supports the observations of PONDER AND ROBINSON[5], who have shown that the haemolytic or critical volume of erythrocytes depends on the composition of the suspending medium.

The sigmoid shape of the haemolysis curve is interpreted as being due to the statistical nature of blood samples. Any sample of normal whole blood contains a proportion of effete cells, which are, for various reasons, more fragile than the remainder of the cell population; it also contains a proportion of more robust cells, those for example which have newly become erythrocytes. The majority of the cells in the sample have characteristics which are intermediate between the two extremes. That part of the curve between 25 % and 75 % may therefore be regarded as the interquartile range of the cumulative frequency distribution of red cell fragility.

The effect of adding solid solute during the main lytic phase

The effect is shown in Fig. 2 of allowing haemolysis to proceed for 10 min at 3.5 atm and 20° and then adding sufficient solid malonamide to raise the osmotic pressure of the solution to 6.5 atm. Immediately following the addition of solid malonamide haemolysis ceases abruptly, the system becomes opaque to the naked eye and there is a considerable lag before haemolysis begins again.

The same pattern of events occurs when that quantity of solid malonamide required to raise the osmotic pressure of the solution to 5.0 atm is added.

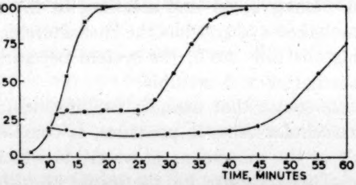

Fig. 2. The effect of adding solid malonamide during the main lytic phase. ×—×, 3.5 atm; ●—●, solid malonamide added 10 min after addition of blood. Final osmotic pressure 6.5 atm.; ○—○, solid malonamide added 11 min after addition of blood. Final osmotic pressure 5.0 atm.

The addition of solid malonamide stops haemolysis abruptly and causes immediate rapid shrinking of the swollen erythrocytes. The prolytic sphere therefore is relatively impermeable to malonamide, for, if this were not so, the addition of solid material—all of which does not dissolve instantaneously—should reduce the rate of haemolysis rather than cause reversal of the process. In terms of classical osmosis the mechanism may be described as follows.

As the added solute goes into solution the extracellular osmotic pressure increases; the resulting osmotic imbalance is corrected by an increase in intercellular solute concentration which is brought about by a reduction in cell water. As the intracellular water leaves, the cells shrink and continue to do so very rapidly until osmotic equilibrium is restored. From this point the slow penetration of malonamide induces swelling once more and the events are repeated until haemolysis recurs.

From these experiments it is evident that the human erythrocyte is permeable to malonamide, but the rate of penetration of this solute is so low relative to water, that it has the property of inducing osmosis.

The effect of temperature change on malonamide induced haemolysis

The effect of temperature change at constant osmotic pressure has been examined and representative experiments are shown graphically in Figs. 3, 4 and 5.

From these results it may be concluded that, at constant osmotic pressure, the rate of haemolysis increases with increasing temperature and, that the effect of temperature change becomes greater as the osmotic pressure of the malonamide solution is increased.

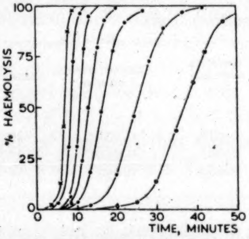

Fig. 3.

Fig. 3. The effect of temperature change at constant osmotic pressure, 3.5 atm. Temperature decreasing from left to right in steps of 5°. ×—×, 3.5 atm. at 35°; ○—○, 3.5 atm. at 5°.

Fig. 4. The effect of temperature change at constant osmotic pressure, 5.0 atm. Temperature decreasing from left to right in steps of 5°. ×—×, 5.0 atm. at 35°; ○—○, 5.0 atm. at 5°.

Fig. 5. The effect of temperature change at constant osmotic pressure, 6.5 atm. Temperature decreasing from left to right in steps of 5°. ×—×, 6.5 atm. at 35°; ○—○, 6.5 atm. at 5°.

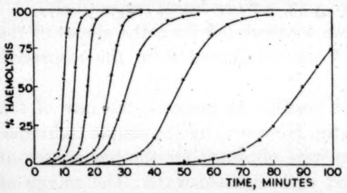

Fig. 4.

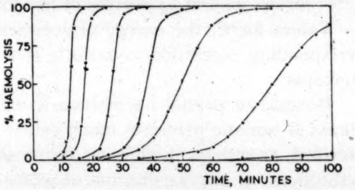

Fig. 5.

The effect of temperature change on the rate of haemolysis at constant osmotic pressure suggested that the data might fit the Arrhenius equation. Although this equation has its origin in the field of chemical kinetics, the exponential law which it expresses describes the effect of temperature change on many rate processes in both physical and chemical systems. In logarithmic form the equation is

$$\log V = \log A - \frac{E}{2.303\,RT}$$

where V is the rate of the process, in appropriate units, at the absolute temperature T and R is the universal gas constant; A is a constant and E is the energy of activation of the process.

For the interpretation of these experiments the rate of haemolysis, V, is defined as the average slope of the haemolysis curve, between 25 % and 75 % haemolysis.

The Arrhenius plot of the data obtained from two series of experiments carried out at different times with blood from the same donor is shown in Fig. 6. From this graph, of slope — 2855, E was found to be 12,970 cal and log A had the value 10.80.

In order to reduce fortuitous error and to allow also for inherent biological variation, the data from a number of similar experiments were subjected to statistical analysis. The parameters of the regression equation

$$\log V = -m\,(1/T) + C$$

were found by the method of least squares; the standard error of estimate and the correlation coefficient were calculated, and the level of significance was assessed by Student's "t" Test, as shown in Table II.

TABLE II

STATISTICAL ANALYSIS OF EXPERIMENTAL DATA

Osmotic pressure (Atm)	*Number of observations*	*Parameters of regression equation*		*Correlation coefficient (—)*	*Standard error of estimate*	*Student's t test*
		-m	*c*			
3.5	41	2815	10.7361	0.978	0.0515	29.21
5.0	22	3482	12.8896	0.981	0.0560	22.66
6.5	14	4236	15.3702	0.965	0.0937	12.70

In each of the three sets of results there is a high degree of negative correlation which is also very highly significant since, at the 0.1 % probability level, the values of "t" for 40, 22 and 14 degrees of freedom are 3.55, 3.79 and 4.14 respectively.

Values for E, the energy of activation, were calculated from the slopes of the corresponding regression equations and $\log A$ was evaluated from the respective intercepts.

Because of partial haemolysis it was not possible to measure the rate of the process at osmotic pressures much below 3.5 atm. However, by the simple technique described, rate data at different temperatures were obtained for the haemolysis of erythrocytes at a 1:21 dilution of whole blood with distilled water. The energy of activation and $\log A$ were evaluated as before and found to be 5,100 cal and 4.60 respectively. The information is summarized in Table III.

TABLE III

EXPERIMENTAL VALUES OF E AND LOG A

Osmotic pressure (Atm)	*Energy of activation (cal)*	*Log A*
0	5,100	4.60
3.5	12,830	10.74
5.0	15,880	12.90
6.5	19,300	15.37

Seeking a correlation between the energy of activation of the process and the osmotic pressure of the haemolysing solution, a graph was drawn, as shown in Fig. 7.

The linear relationship derived from this graph may be expressed

$$E = 2{,}180\,\pi + 5{,}100$$

The value of 5,100 cal for the intercept is of considerable interest since the activation energy for the self-diffusion of water is near this value, being quoted by MOELWYN-HUGHES[6] as 4,400 cal and by ORR AND BUTLER[7] as 5,300 cal.

There is also a linear relationship between log A and the osmotic pressure of the haemolysing system,

$$\log A = 1.7\,\pi + 4.60$$

Thus, if the osmotic pressure in atmospheres, between 0° and 35°, of a hypotonic aqueous solution of malonamide is known, the activation energy and log A may be derived from the two equations given above; by substituting the derived values in the Arrhenius equation, the rate of haemolysis may be calculated for any temperature within the range quoted. A comparison between observed and calculated rates of haemolysis is illustrated in Table IV.

Considering the number of variables involved, the agreement is fair. By applying the Arrhenius equation therefore, a considerable body of experimental data has been summarised in convenient form.

Since E and log A are each linearly related to the osmotic pressure of the solution, it might be expected that they should have a linear relationship with each other; that this is so is shown in Fig. 8.

TABLE IV

COMPARISON OF OBSERVED AND CALCULATED VALUES FOR THE RATE OF HAEMOLYSIS

Osmotic pressure (Atm)	*Temperature °C*	*Rate of haemolysis*	
		(obs.)	*(calc.)*
0	0	2.7 %/sec	3.3 %/sec
0	35	9.1 %/sec	10.3 %/sec
0	20	6.3 %/sec	6.3 %/sec
2.3	20	9.1 %/min	7.9 %/min
2.4	30	16.7 %/min	14.1 %/min
3.5	10	5.6 %/min	5.6 %/min
3.5	30	31.2 %/min	24.9 %/min
3.5	35	40.7 %/min	43.5 %/min
5.0	30	28.2 %/min	37.3 %/min
6.5	30	20.0 %/min	28.8 %/min

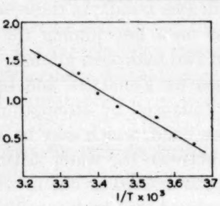

Fig. 6.

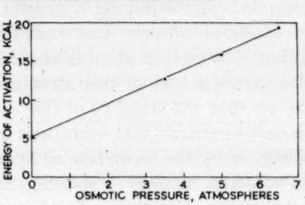

Fig. 7.

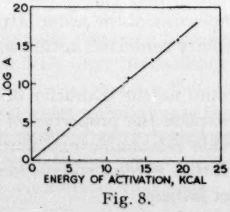

Fig. 8.

Fig. 6. Graph of log V on $1/T$ for blood from a single donor. –m, 2855; E, 12,970 cal.; log A, 10.80. ●—●, Expt. 1; ○—○, Expt. 2.

Fig. 7. The relationship between the energy of activation and the osmotic pressure of the solution.

Fig. 8. The relationship between log A and the energy of activation.

The interesting feature of this graph is that the equation of the line passes through the origin. The interpretation of this relationship involves the consideration of other factors which are better discussed in a later section.

DISCUSSION

The structure of water

The experiments have been described in terms of classical osmosis and although some conclusions are possible, the mechanism remains obscure. It would therefore seem pertinent to reconsider the facts in the light of modern theories of the structure of water.

It has been appreciated for many years that the unique properties of water depend largely on the association of its molecules. BERNAL AND FOWLER[8], however, were first to postulate that an ice-like, orderly arrangement of the molecules persisted in the liquid state; although later work has shown the need for detail modifications, their general picture of extensive tetrahedral co-ordination remains valid.

It is not within the scope of this paper to review the work of the many individuals who have contributed to the understanding of water structure, but mention must be made of FORSLIND's water model[9], and his discussion of the problem.

From a study of the wave mechanical aspects of the water molecule FORSLIND has developed a structural model of water which has proved most useful in interpreting the experiments reported here. This author has shown that the electronic distribution of the oxygen valence state gives rise to hybrid bond maxima which are approximately tetrahedrally directed and further, that the formation of a water molecule causes only slight modification of the oxygen hybridisation. Thus, while the water molecule is usually depicted as an oxygen atom with two hydrogens at an angle of 104.5°, there are also, in the plane perpendicular to the first, two unfilled bonds at an angle of 131.8° (see ref. 9). Since the true tetrahedral angle is 109.5°, the formation is possible of an extensive, though not ideal, tetrahedrally co-ordinated structure, which depends on the linking of oxygen atoms by hydrogen bonds; in these bonds the proton oscillates between two equilibrium positions on a line joining the oxygen nuclei, but each oxygen atom is associated only with two hydrogen atoms.

The consequences of this structure are discussed by FORSLIND and it suffices here to say that the stability of the water lattice is enhanced by attenuation of the anharmonic intermolecular vibrations of the hydrogen bond, which may be achieved by cooling, or by the formation of hydrogen bonds between the water lattice and a specific substrate surface. A specific substrate surface is defined as one in which the molecular surface groups are of such a nature and so placed that hydrogen bonding is possible without undue deformation of the water lattice; that is, the better the fit between the substrate surface groups and the basal molecules of the water lattice, the greater the stability conferred on the lattice. The stability conferred in this way may extend 300 Å and more from the surface concerned.

FORSLIND has employed this water model to account for the hydration of certain clay sols[10] and JACOBSON[11] has used the concept to explain the properties of deoxyribonucleic acid solutions. Since both authors provide reasonable explanations of these phenomena, it seems not unreasonable to suggest that, given a specific molecular structure, small molecules also will stabilise the water lattice.

Water stabilished in this way, in the vicinity of a small molecule, is not true water of hydration; it is, rather, a zone in which there are more intact hydrogen bonds than in the body of the liquid. Because of the more extensive hydrogen bonding within the stabilized zone the water molecules remain longer in their positions in the lattice and so have less freedom of movement than the molecules outside the zone.

It will be shown in the following sections that the concept of water structure, and its consequences provide a reasonable explanation of the experiments reported here.

The movement of water in blood

Before considering possible mechanisms in detail it is worth recalling that osmotic equilibrium is a dynamic state in which there is a continuous exchange of water across a semipermeable membrane. This dynamic exchange, or turnover has been demonstrated in normal blood by GOVAERTS AND LAMBRECHTS[12], who found that HDO added to whole blood is equally distributed between the cells and plasma within 1 min. There is no reason for supposing that the osmotic equilibrium attained in aqueous malonamide solutions is in any way different from this.

When whole blood is diluted with hypotonic aqueous malonamide there occurs the immediate redistribution of water which restores osmotic equilibrium and induces cell swelling. In the same way there is an immediate redistribution of water—which induces cell shrinkage—when haemolysis is arrested by adding solid solute to the haemolysing system. There is, in these two statements, the vaguely implied notion that water is drawn across the semipermeable membrane into the more concentrated solution. This is not necessarily true, however, because the effect—swelling or shrinking—would be the same if the volume of water required to maintain osmotic equilibrium was merely retained on the more concentrated side of the membrane. The experiments are re-examined below with this point in mind.

With regard to the addition of solid malonamide, the instant before the solid material is added the cells are swollen and in osmotic equilibrium with the solution; water is entering and leaving the cells at the same rate. The addition of solid malonamide to the extracellular side of the membrane could, by increasing the stability of the extracellular water lattice, reduce or even stop the flow of water into the cell. In this circumstance water would continue to leave the cell until a new dynamic osmotic equilibrium is attained. Thus the addition of solid solute to the extracellular phase causes the cells to shrink, not by withdrawing water, but by retaining in the extracellular phase that volume of water necessary to maintain the original osmotic equilibrium.

The same mechanism could account for the haemolysis of erythrocytes when blood is diluted with water. With sudden dilution of the plasma the outgoing cell water virtually ceases to flow, but the ingoing water continues and may do so at an increased rate, since the stability of the extracellular water lattice is reduced. If the dilution of the plasma is sufficiently great the combined effects of water retention and continued water uptake cause the swelling which culminates in haemolysis. The idea that the swelling or shrinking of erythrocytes depends on the structural integrity of the extracellular phase suggests that the rate of haemolysis may be correlated with the viscosity of that phase.

The energy of activation

It has been shown that a linear relationship exists between the energy of activation of the process being measured and the osmotic pressure of the haemolysing solution and that this relationship holds good to zero osmotic pressure. The explanation offered for the behaviour of erythrocytes when whole blood is diluted with water points to a connection between the observed effect and viscous flow. Viscosity is a measure of resistance to flow and decreases with increasing temperature, whereas the rate of haemolysis increases with increasing temperature; fluidity, which measures tendency to flow and increases with increasing temperature, is more closely related to the experimental observations.

It would appear then, that the energy of activation derived from observations on the effect of temperature change on water induced haemolysis refers to the fluidity of water. This possibility has been examined by determining E and $\log A$ for the fluidity of water in the temperature range 5°–35°. The graph of $\log \phi$ on $1/T$ is slightly convex to the x-axis but, for the purpose of this work, was taken as linear. The energy of activation found in this way was 4,200 cal and $\log A$ has the value 4.14; these values compare not too unfavourably with 5,100 cal and 4.60 obtained by experiment for water induced haemolysis. By extrapolating the E/π and $\log A/\pi$ graphs a further 0.2 atm, thereby allowing for the contribution of the plasma solutes to the osmotic pressure of the haemolysing system, the agreement is quite striking, for E then becomes 4,300 cal and $\log A$ has the value 4.25.

The existence of a linear relationship between the energy of activation and the osmotic pressure of the solution suggests that the process upon which E depends is essentially the same throughout the range of the experiments, namely the effect of temperature change on the fluidity of the extracellular phase.

On re-examining malonamide induced haemolysis the following picture emerges. When whole blood is added to a hypotonic malonamide solution there is considerable dilution of the plasma; the extracellular water lattice stability is reduced and so the fluidity of the extracellular phase is increased. Up to the point of addition the cells were in dynamic osmotic equilibrium with the plasma. The decrease in lattice stability of the extracellular water—but not the intracellular water—results in the whole or partial retention of water within the cell, together with an increased flow of water from the solution into the cell. Dynamic osmotic equilibrium is re-established when water is once again entering and leaving the cell at the same rate. As shown experimentally the establishment of this new equilibrium, which accounts for the initial swelling, is extremely rapid and takes place in a few seconds. Water continues to exchange between the cell and the solution until the new equilibrium is disturbed by the penetration of a molecule of malonamide. The loss of a molecule of malonamide has a negligible effect on the stability of the extracellular water lattice, but the effect of that molecule on the relatively small volume of the intracellular phase is considerable in comparison. The cell therefore, retains a proportion of its outgoing water, which is incorporated in the stablised intracellular water lattice, and osmotic equilibrium is restored by a compensating increase in the ingoing water from the solution. Each time a molecule of malonamide enters the cell a proportion of the outgoing equilibrium water is retained and osmotic balance is restored by additional water moving in from the extracellular phase. The ultimate stage in the process is haemolysis and it is evident that the rate of swelling and therefore of haemolysis is governed mainly by the fluidity of the extracellular phase. If the extracellular phase is highly fluid, as it becomes when whole blood is diluted 1:21 with water, haemolysis is very rapid indeed. When the extracellular phase is less fluid, through stabilisation of the water lattice by solute, the rate of haemolysis is less. This accounts for the relationship between the energy of activation and the solution osmotic pressure, since the lower the energy of activation the more rapid the process.

In terms of the collision theory of reaction rates the Arrhenius activation energy refers to kinetic energy only. Later work, however, associates E with potential energy in that it is the measure of a potential energy barrier to the fulfilment of a rate process. The activation energy derived in these experiments refers to the fluidity of the extracellular phase, so E could be considered a measure of the potential energy of the water lattice, since it represents the energy which must be expended before a molecule of water becomes completely detached from its neighbours and attains the active state for flow. The fact that the activation energy increases with increasing osmotic pressure shows that, within the range of these experiments, the potential energy or stability of the water lattice increases with increasing solute concentration.

The entropy of activation

For the further evaluation of the results it is necessary to interpret the non-exponential factor of the Arrhenius equation, because E depends only on the effect of heat on the process, and the factor A covers all the remaining variables.

In a consideration of the temperature dependence of viscosity ANDRADE[13] interprets A as a measure of the rate of the process at infinite temperature. While infinite temperature as such has no meaning for real systems, the artifice does suggest that A represents the rate of the process in the absence of intermolecular forces.

The theory of absolute reaction rates has been applied to viscous flow[14], and the equation derived has, for fluidity, the form

$$\phi = \frac{h\mathrm{N}}{V} \mathrm{e}^{\Delta S^{*}/R}\, \mathrm{e}^{-\Delta H^{*}/RT}$$

where N is the Avogadro number, h is Planck's constant and V is the molar volume of the liquid; ΔS^{*} is the entropy of activation and ΔH^{*} is the heat of activation.

Since ΔH^{*} is very nearly the same as E, the experimental activation energy, when the Arrhenius equation and this one are compared, it is seen that

$$\log A = \log \frac{h\mathrm{N}}{V} + \frac{\Delta S^{*}}{2.303\, R}$$

Log A is therefore, a function of the entropy of activation of the process.

The process of activation therefore, involves an increase in the randomness of the system; this is in agreement with the supposition that liquid water has an extensive quasi-crystalline structure, for, if the unit of flow is the individual water molecule, activation for flow necessitates the breaking of hydrogen bonds and disruption of the structure. The linear relationship between log A and the osmotic pressure of the solution shows that the stability of the water lattice increases with increasing solute concentration, since the more ordered the initial structure the greater the number of bonds to be broken in order to attain the active state.

The linear relationship between log A and E confirms the interpretation already suggested. It is to be expected that lowering of the potential energy of the water lattice will loosen the structure and increase the entropy of the system; the entropy of activation would therefore decrease. At zero activation energy, which occurs at infinite temperature, the system is completely random in the initial state and so the entropy of activation is also zero.

The free energy of activation

Numerical values of ΔS^* were calculated from the logarithmic form of the above equation. Using the thermodynamic equation,

$$\Delta F^* = \Delta H^* - T\Delta S^*$$

and substituting the experimental values of E for ΔH^*, the free energy of activation, ΔF^*, was calculated for the different osmotic pressures employed. This information is tabulated below.

TABLE V

THE ENTROPY AND FREE ENERGY OF ACTIVATION

Osmotic pressure (Atm)	*E (cal)*	*ΔS^* (Cal deg^{-1}mole^{-1})*	*ΔF^* (cal)*
0	5,100	35.5	—5,300
3.5	12,830	63.6	—5,800
5.0	15,880	73.4	—5,630
6.5	19,300	84.8	—5,540

This table is particularly interesting for it shows that the free energy of activation is negative, relatively small and, within the limits of these experiments, virtually constant irrespective of the concentration of the solution. The small negative value of the free energy of activation accounts for the fact that, even with the comparitively high activation energies found at higher osmotic pressures, haemolysis in the main lytic phase occurs spontaneously and rapidly at room temperature. That the free energy of activation is small and negative is the result of the compensating effect of the relatively large positive entropy of activation; as already pointed out this implies the existence of an extensive orderly arrangement when the system is in the initial state, even at zero osmotic pressure. The constant value of the free energy of activation shows that the driving force in the co-ordinate of reaction, in this case fluid flow, is the same whatever the concentration of the solution; it is likely therefore that the mechanism will be the same also and that the unit of flow is the single water molecule.

As suggested at the beginning of the discussion, the concept of water as an ordered lattice structure opens up a new approach to the mechanism of haemolysis. In these experiments at least, the study of the kinetics of the process strongly supports the view that the stabilizing effect of the solute on the water lattice is the principal factor in the mechanics of malonamide induced haemolysis.

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REFERENCES

[1] E. B. HENDRY, *J. Gen. Physiol.*, 35 (1952) 605.
[2] S. GLASSTONE, *Text Book of Physical Chemistry*, 2nd ed. D. Van Nostrand Company, Inc., New York, 1946, p. 672.
[3] E. B. HENDRY, *Edinburgh Med. J.*, 56 (1949) 320.
[4] J. W. CLEGG AND E. J. KING, *Brit. Med. J.*, 2 (1942) 329.
[5] E. PONDER AND E. J. ROBINSON, *J. Physiol. (London)*, 83 (1934) 34.
[6] E. A. MOELWYN-HUGHES, *The Kinetics of Reactions in Solution*, O.U.P. Oxford, 1933.
[7] W. J. C. ORR AND J. A. V. BUTLER, *J. Chem. Soc.*, (1935) 1273.
[8] J. D. BERNAL AND R. H. FOWLER, *J. Chem. Phys.*, 1 (1933) 515.
[9] E. FORSLIND, *Proc. Swed. Cement and Concrete Research Inst.*, No. 16 (1952).
[10] E. FORSLIND, *Proc. Swed. Cement and Concrete Research Inst.*, No. 17 (1952).
[11] B. JACOBSON, *Nature*, 172 (1953) 666.
[12] J. GOVAERTS AND A. LAMBRECHTS, *Nature*, 157 (1946) 301.
[13] E. N. DA C. ANDRADE, *Phil. Mag.*, 17 (1934) 698.
[14] S. GLASSTONE, K. J. LAIDLER AND H. EYRING, *The Theory of Rate Processes*, McGraw Hill, New York, 1941, 484.

THE CONSEQUENCES OF SYSTEMATIC ERROR IN ENZYME KINETICS

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SUMMARY

The consequences of systematic error in enzyme kinetics was considered by introducing a first order error term into the MICHAELIS–MENTEN–BRIGGS–HALDANE equation. The situations investigated were those involving over- or undercorrection of an enzyme or substrate blank reaction in the absence and presence of product inhibition and in the presence of a totally competitive, non-competitive or uncompetitive inhibitor. In addition, consideration was given to errors arising from a departure from the BEER-LAMBERT relationship and those proportional to the velocity or substrate concentration. Finally, attention has been called to the questionable validity of using weighting procedures to correct for random error in the presence of systematic error.

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* Contribution No. 2560.

INTRODUCTION

The apparent initial rate of many enzyme catalyzed reactions is given by eqn. 1,*

$$v = \mathrm{d}[\mathrm{P}]/\mathrm{d}t = -\mathrm{d}[\mathrm{S}]/\mathrm{d}t = k[\mathrm{E}]\,[\mathrm{S}]/(K + [\mathrm{S}]) \qquad (1)$$

provided [E] and [S] are varied over a sufficiently limited range and all other reaction parameters are held constant. In this communication we shall be concerned initially with the consequences of a systematic error which will introduce an additional first order term into eqn. 1 to give eqn. 2.

$$v = k[\mathrm{E}]\,[\mathrm{S}]/(K + [\mathrm{S}]) + k_{\mathrm{A}}[\mathrm{A}] \qquad (2)$$

It is assumed in eqn. 2 that A will yield products which are indistinguishable from those arising from S.

When [E] is invarient, eqn. 1 is linear and eqn. 2 non-linear with respect to $1/v$ *vs.* $1/[\mathrm{S}]$, $[\mathrm{S}]/v$ *vs.* [S] or v *vs.* $v/[\mathrm{S}]$ (see ref. 1). Thus, it might be argued that the presence of a systematic error, of the type contemplated in eqn. 2, would necessarily be revealed by a non-linear relationship between the two members of any one of the above three pairs of parameters and that the only hazard would be one of confusing a situation described by eqn. 2 with one devoid of systematic error but characterized by a dependency upon [S] other than that associated with eqn. 1. However, this view ignores the fact that many enzymic studies are conducted over such a limited range of [S] that an existing non-linear relationship may not be revealed, with the result that k and K will be evaluated on the basis of an apparent linear relationship which in fact may vary with the relative magnitudes of [S] and K.

To illustrate the kinetic consequences of several of the more common systematic errors, eqn. 2 was evaluated using assumed but reasonable values for the various constants and independent variables and the results so obtained were compared with those arising in the absence of systematic error or errors. The first cases considered were those involving the so-called enzyme and substrate blanks.

It frequently is observed that products indistinguishable from those formed in a total system, otherwise described by eqn. 1, will arise from either or both E and S when examined separately. In practice the rates of these latter reactions may be individually determined and then subtracted from the rates observed for the total system**. Thus, the term k_{A} [A] in eqn. 2 may be specified as in Table I to give eqns. 2_a to 2_f***. These latter equations were evaluated for $K = 5.0 \cdot 10^{-2}\,M$, $k = 2.0 \cdot 10\ M/\mathrm{min}/M$, $\pm k_{\mathrm{A}_1} = \pm k_{\mathrm{A}_2} = \pm 2.0 \cdot 10^{-1}\ M/\mathrm{min}/M$, $\pm k_{\mathrm{A}_3} = \pm 2.0 \cdot 10^{-4}\ M/\mathrm{min}/M$, $[\mathrm{E}] = 5.0 \cdot 10^{-5}\,M$ and $[\mathrm{S}] = 5.0 \cdot 10^{-4}\,M$ to $5.0\,M$. The data so obtained are presented in Figs. 1a, 1b and 1c in the form of v *vs.* $v/[\mathrm{S}]$plots.

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[*] Where [E] and [S] are the initial enzyme and substrate concentrations and k and K are the two constants derivable from the dependence of v upon [S] when [E] is invarient. For a discussion of the procedures involved see ref. 1.

[**] For a discussion of the validity of this procedure see ref. 2.

[***] Although eqns. 2_a and 2_b may appear to be equivalent to equations 2_b and 2_d it will be shown subsequently that the addition of $\pm k_{\mathrm{A}_1}$ $[\mathrm{E_T}]$ to eqn. 1. leads to different rate equations than does the addition of $\pm k_{\mathrm{A}_2}$ $[\mathrm{E_F}]$. This situation arises from the fact that in the former instance it is assumed that all of the enzyme present, $[\mathrm{E_T}]$, can give rise to a blank reaction whereas in the latter only the free enzyme, $[\mathrm{E_F}]$, can so react. While these pairs of rate equations are different they are kinetically indistinguishable so long as the conditions implicit in eqn. 1 are maintained.

It is evident from Figs. 1a, 1b and 1c that the systematic errors considered in Table I will lead to errors in the direction indicated in Table II. The information summarized in Table II may be interpreted in several ways. First, if a non-linear plot is observed it follows from Table II that the situations described by eqns. 2_a, 2_b and 2_e will simulate activation by excess substrate, in the sense used by WOLF AND NIEMANN[3], and that of equation 2_f of inhibition by excess substrate[*]. Alternatively, if an apparent linear relationship is observed, over a narrow range of [S], constants evaluated on the basis of such a relationship will tend to be in error in the directions indicated in Table II. Finally, the data summarized in Table II are useful in identifying those errors which tend to be predominantly additive, *e.g.*, a combination of 2_c and 2_f, or of 2_d and 2_f. There is no combination involving both enzyme

TABLE I

SYSTEMATIC ERRORS ASSOCIATED WITH ENZYME OR SUBSTRATE BLANKS

Eqn.	*Source of error*	$k_A{}^*$	*[A]*	*Nature of error*
2_a	Enzyme blank	k_{A_1}	$[E_T]^{**}$	Undercorr. for blank
2_b	Enzyme blank	k_{A_2}	$[E_F]^{***}$	Undercorr. for blank
2_c	Enzyme blank	$-k_{A_1}$	$[E_T]^{**}$	Overcorr. for blank
2_d	Enzyme blank	$-k_{A_2}$	$[E_F]^{***}$	Overcorr. for blank
2_e	Substrate blank	k_{A_3}	$[S]^{\S}$	Undercorr. for blank
2_f	Substrate blank	$-k_{A_3}$	$[S]^{\S}$	Overcorr. for blank

[*] It is assumed that in every case the rate of reaction will be first order in [A].

[**] Total enzyme concentration, *i.e.*, $[E_T] = [E]$.

[***] Free enzyme concentration, *i.e.*, $[E_F] = [E_T] - [ES]$.

[§] Free substrate concentration. However, this will be equivalent to the initial or total substrate concentration, *i.e.*, $[S_T] \doteq [S]$, everywhere except in eqn. 3 where $[S_T] \doteq [S] + [P]$. The conditions are necessary for the validity of eqn. 1.

TABLE II

RELATION BETWEEN TRUE AND APPARENT VALUES OF K AND k

$k_A[A] =$	$k_{A_1}[E_T]$ $(2_a)^*$	$k_{A_2}[E_F]$ $(2_b)^*$	$-k_{A_1}[E_T]$ (2_c)	$-k_{A_2}[E_F]$ (2_d)	$k_{A_3}[S]$ $(2_e)^*$	$-k_{A_3}[S]$ $(2_f)^{**}$
K'^{***}	$< K$	$< K$	$> K$	$> K$	$> K$	$< K$
$k'^{\S}$	$\lessgtr k$	$< k$	$\gtrless k$	$> k$	$> k$	$< k$

[*] Simulates activation by excess substrate.

[**] Simulates inhibition by excess substrate.

[***] Apparent value of K.

[§] Apparent value of k.

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[*] See ref. 1, p. 81 for the characteristics of inhibition by excess substrate. Cases 2_e and 2_f are of particular interest because of the requirement of relatively high values of [S] for the demonstration of either activation or inhibition by excess substrate. In these cases it is important that the substrate blank be evaluated with considerable precision because if it is overestimated inhibition by excess substrate will be simulated and if underestimated activation by excess substrate will be mimicked. In eqns. 2_a and 2_b the dependency of v upon [S] is the same as that encountered in the case of introduction of an endogenous substrate with the enzyme preparation[4]. However, in the latter instance the dependency of v upon [E] differs from that of equations 2_a and 2_b.

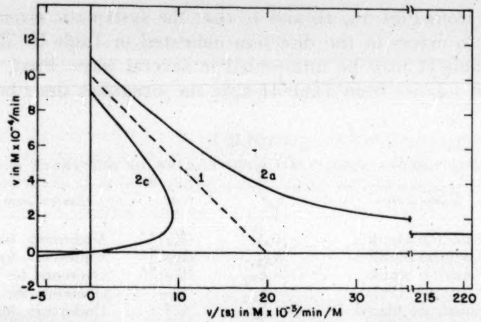

Fig. 1a. Systematic error effects on eqn. 1: eqns. 2_a and 2_c with $\pm k_{A_1} = \pm 2.0$ *M*/min/*M* to exaggerate the effects of the error term. Eqns 2_a and 2_c have asymptotes at $v = k_{A_1}[E_T]$ and $v = -k_{A_1}[E_T]$ and intercepts at $v = (k + k_{A_1})[E_T]$ and $v = (k - k_{A_1})[E_T]$ respectively.

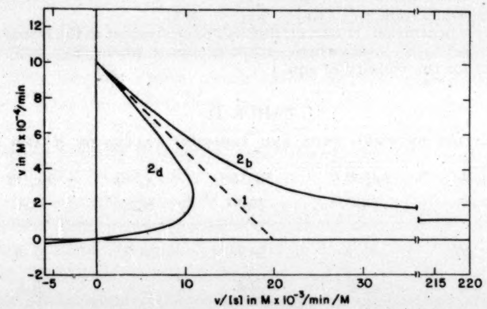

Fig. 1b. Systematic error effects on eqn. 1: eqns. 2_b and 2_d with $\pm k_{A_2} = \pm 2.0$ *M*/min/*M* to exaggerate the effects of the error term. Eqns. 2_b and 2_d have asymptotes at $v = k_{A_2}[E_T]$ and $v = -k_{A_2}[E_T]$ respectively and a common intercept at $v = k[E_T]$.

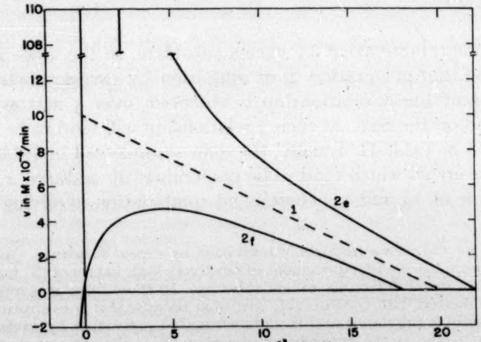

Fig. 1c. Systematic error effects on eqn. 1: eqns. 2_e and 2_f with $\pm k_{A_3} = \pm 2.0\cdot 10^{-3}$ *M*/min/*M* to exaggerate the effects of the error term. Eqns. 2_e and 2_f have asymptotes at $v/[S] = k_{A_3}$ and $v/[S] = -k_{A_3}$ and intercepts at $v/[S] = k_{A_3} + k[E_T]/K$ and $v/[S] = -k_{A_3} + k[E_T]/K$ respectively.

and substrate errors that will be generally compensatory because errors arising from improper treatment of the enzyme blank will be dominant at low values of [S] and those associated with the substrate blank at high values of [S].

An alternative procedure for interpreting the kinetic consequences of systematic errors of the above kind, which is also useful for assessing the significance level of the individual errors, is based upon transposition of equations 2_a to 2_f to equations $2'_a$ to $2'_f$ inclusive.

$$v\,[\mathrm{S}] + vK = (k + k_{\mathrm{A}_1})\,[\mathrm{E_T}]\,[\mathrm{S}] + k_{\mathrm{A}_1}\,[\mathrm{E_T}]\,K \qquad (2'_a)$$

$$= k\,[\mathrm{E_T}]\,[\mathrm{S}] + k_{\mathrm{A}_2}\,[\mathrm{E_T}]\,K \qquad (2'_b)$$

$$= (k - k_{\mathrm{A}_1})\,[\mathrm{E_T}]\,[\mathrm{S}] - k_{\mathrm{A}_1}\,[\mathrm{E_T}]\,K \qquad (2'_c)$$

$$= k\,[\mathrm{E_T}]\,[\mathrm{S}] - k_{\mathrm{A}_2}\,[\mathrm{E_T}]K \qquad (2'_d)$$

$$= (k\,[\mathrm{E_T}] + k_{\mathrm{A}_3}\,K)\,[\mathrm{S}] + k_{\mathrm{A}_3}\,[\mathrm{S}]^2 \qquad (2'_e)$$

$$= (k\,[\mathrm{E_T}] - k_{\mathrm{A}_3}\,K)\,[\mathrm{S}] - k_{\mathrm{A}_3}\,[\mathrm{S}]^2 \qquad (2'_f)$$

Examination of these latter equations readily discloses how the various systematic errors interact with k and K. Furthermore, division of both members of equations $2'_a$ to $2'_f$ by $(K + [\mathrm{S}])$ reveals that systematic errors of the kind under consideration can be ignored only if $v \gg k_{\mathrm{A}_1}\,[\mathrm{E_T}]$ or $k_{\mathrm{A}_1} \ll k\,([\mathrm{S}]/(K + [\mathrm{S}]))$ for cases 2_a and 2_c, $v \gg k_{\mathrm{A}_2}\,[\mathrm{E_T}]\,\,(K/(K + [\mathrm{S}]))$ or $k_{\mathrm{A}_2} \ll k\,([\mathrm{S}]/K)$ for cases 2_b and 2_d and $v \gg k_{\mathrm{A}_3}\,[\mathrm{S}]$ or $k_{\mathrm{A}_3} \ll k\,[E_{\mathrm{T}}]/(K + [\mathrm{S}])$ for cases 2_e and 2_f.

It has been suggested upon several occasions that evaluations based upon eqn. 1 should be weighted to compensate for the altered error function arising from transformation of a hyperbolic to a linear function[5]. While this practice may be justified for data containing only random error the question arises as to its suitability for data containing both random and systematic errors. Values of k and K were obtained from equations 2_a to 2_f for several tenfold ranges in [S] using both weighted and unweighted linear least-squares fits of lines based upon $1/v$ *vs.* $1/[\mathrm{S}]$ plots. It was found that either procedure could lead to values of k and K which were closer to the true values depending upon the particular range of [S] investigated. However, when equations 2_e and 2_f were employed the unweighted fits generally but not invariably led to more accurate values of k and K than did the weighted fits. Thus, it is clear that the practice of weighting to accommodate random error, when both random and systematic errors are present, is no panacea and if used must be used with discretion to avoid causing greater errors in the values of the kinetic constants than would obtain were it not used.

Eqn. 2 may be extended to accommodate situations involving reversible inhibition by reaction products or added inhibitor[1,6]. In the former instance eqn. 2 may be modified to give eqn. 3.

$$v = \{k\,(K_1/(K_1 - K))\,[\mathrm{E}]\,[\mathrm{S}]\}/\{K((K_1 + [\mathrm{S_T}])/(K_1 - K)) + [\mathrm{S}]\} + k_{\mathrm{A}}\,[\mathrm{A}] \qquad (3)$$

Evaluation of eqn. 3, with k_{A} [A] defined as in Table I, $K_1 = 2K$, K and $0.5\,K$, $[\mathrm{S_T}] = K$ and with the values of k, K, $\pm\,k_{\mathrm{A}_1}$, $\pm\,k_{\mathrm{A}_2}$, $\pm\,k_{\mathrm{A}_3}$ and [E] used previously, was based upon the treatment of FOSTER AND NIEMANN[7–12]. In principle, the integral $\int_0^t$ (eqn. 3) dt was evaluated for $k_{\mathrm{A}}\,[\mathrm{A}] = 0$, to obtain values of t for arbitrarily chosen extents of reaction of 20 and 40 %. The integral $\int_0^t$ (eqn. 3) dt was then evaluated for values of t corresponding to those above to obtain apparent values of [S′] that differed from those of [S] because of contributions arising from the term k_A [A]. The slopes of $([S_o] - [S_t])/t$ *vs.* $(\ln([S_o]/[S_t]))/t$ and $([S_o] - [S'_t])/t$ *vs.* $(\ln([S_o]/[S'_t]))/t$ plots[7–12], which were but slightly curved in the latter instance, were then used to arrive at an estimate of the apparent values of K'_1 relative to those of K_1, for which $k_A [A] = 0$. It was found that for equations 3_a, 3_b, and 3_e $K'_1 > K_1$ and for 3_c, 3_d and 3_f $K'_1 < K_1$. In other words, systematic error arising from no or under-correction of the enzyme or substrate blank reactions could lead one to over-estimate the magnitude of an apparent enzyme–product dissociation constant whereas over-correction of either of the two blank reactions could lead to the opposite result.

For an added inhibitor, eqn. 2 may be transformed into eqns. 4, 5 and 6, which may be associated with "totally competitive", "totally non-competitive"

$$v = \{k\,[E]\,[S]\}/\{K\,(1 + [I]/K_2) + [S]\} + k_A\,[A] \quad (4)$$

$$v = \{k\,[E]\,[S]/(1 + [I]/K_2)\}/\{K + [S]\} + k_A\,[A] \quad (5)$$

$$v = \{k\,[E]\,[S]\}/\{K + [S]\,(1 + [I]/K_2)\} + k_A\,[A] \quad (6)$$

and "totally uncompetitive" inhibition respectively[1]. Eqns. 4, 5 and 6 were evaluated for the values of k, K, $\pm k_{A_1}$, $\pm k_{A_2}$, $\pm k_{A_3}$, [E] and [S] used previously but with $(1 + [I]/K_2) = 1.0$, 1.1, 2.0 and 10.0 respectively. The data so obtained were presented in $1/v$ *vs.* $1/[S]$ plots to produce six sets of curves, each set consisting of a family of four curves. When examined over a sufficiently wide range of [S] the non-linearity of each curve was readily apparent. However, in contrast to the case involving only [S] as the independent variable, *vide ante*, it did not appear to be worth while to attempt to identify any of the above curves with those arising in the absence of a systematic error from a dependency upon [S] and [I] other than that assumed in eqns. 4, 5 and 6 principally because situations involving a simultaneous dependency upon $[S]^n$ and $[I]^m$ are largely unexplored. Instead, attention was directed to the more immediate question as to the possibility of confusing one type of inhibition with another by examining a system over a sufficiently narrow range of [S] as to be led

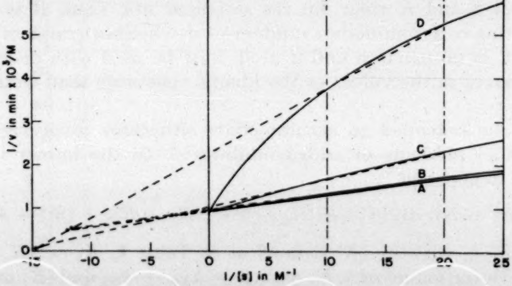

Fig. 2. Simulation of mixed inhibition by a "totally competitive" inhibitor: solid lines, eqn. 4_a with $k_{A_1} = 2\ M/\text{min}/M$ to exaggerate the effects of the error term and with $(1 + [I]/K_2) = 1$, 1.1, 2 and 10 for curves A, B, C and D respectively; broken lines, extrapolated cords of eqn. 4_a between $1/[S] = 20$ and $10\ M^{-1}$.

to the conclusion that the dependence of v upon [S] and [I] was linear rather than non-linear. For each family of four curves two or more ranges of [S], each entailing a two-fold variation in [S], were selected as to encompass as large a portion of each curve without leading to negative values of v. Each of the four chords of the line segments associated with a particular range in [S] was extrapolated to its intersection with the other three chords as illustrated in Fig. 2. These points of intersection then were examined to determine their ability to simulate a single point of intersection and to determine their proximity to the $1/v$ and $1/[S]$ axes. This procedure was repeated for all other ranges of [S] and for the other five sets of curves. This information, interpreted in terms of type of inhibition simulated is given in Table III.

TABLE III

SIMULATION OF ALTERNATIVE TYPES OF INHIBITION

As the significance of the error term $k_A[A]$ diminishes, simulation of alternative types of inhibition become less probable. However, even when this element of confusion becomes unimportant errors in the magnitudes if k, K and K_2 may persist.

Eqn.	$k_A[A]$	$k_{A_1}[E_T]$	$k_{A_2}[E_F]$	$-k_{A_1}[E_T]$	$-k_{A_2}[E_F]$	$k_{A_3}[S]$	$-k_{A_3}[S]$
4	"C"*	M**, (N)***	M**	M**	M**	M**	M**, (N)***
5	"N"§	M**	N§§	M**, (C)***, (N)***	N§§	M**, N§§	M**, N§§
6	"U"§§§	X✱	X✱	M**	M**	M**, (N)***	X✱

* "Totally competitive" with systematic error.
** Inhibition of mixed types, *i.e.*, combination of partially competitive and partially non-competitive, etc.
*** Fortuitous simulation for a singular range of [S], or more accurately $[S]/K$.
§ "Totally non-competitive" with systematic error.
§§ No change in type but only in magnitude of k, K and K_2.
§§§ "Totally un-competitive" with systematic error.
✱ No currently recognized type.

It is evident from these data that situations interpreted as involving "totally non-competitive" inhibition require careful scrutiny in order to be certain that the type of inhibition and/or the magnitude of the inhibition constants are not a consequence of a systematic error arising from improper treatment of the enzyme or substrate blank reactions of a system which in fact may be "totally competitive" or "totally non-competitive" in basic character.

In the preceding discussion particular attention has been paid to systematic errors arising from enzyme or substrate blank reactions because such reactions are a common feature of many enzyme catalyzed reactions. In particular situations other systematic errors may arise. For example, if the specific substrate or reversible inhibitor is in facile equilibrium with a form, *e.g.*, a micelle, which interacts with the enzyme to a lesser degree than the monomer, K or K_2 will be over-evaluated since the observed values of v will exhibit the same dependencies upon [S] and [I] as in eqns. 1 and 4 with k_A $[A] = 0$. Alternatively, if a systematic error is present which is proportional to v, *i.e.*, eqn. 2 with k_A $[A] = k_{A_4} v$, then only the value of k will be influenced and the plots will remain linear. An example of this situation would be a systematic error in the time scale. Similarly, a systematic error in the determination of [S] which is proportional to [S] will be reflected only in the value of K. Finally, when a reaction is followed spectrophotometrically by measuring $-d\,[S]/dt$ or $d[P]/dt$, most departures from the BEER-LAMBERT relationship are representable by an equation of the form $[C]_{obs.} = [C]_{act.} \pm k_B\,[C]^n$ which can be differentiated to $d[C]obs./dt = d[C]act./dt \pm k_B\,n\,[C]I^{-1}\,d[C]/dt$. When $n = 1$, the systematic error will be proportional to v and, hence, reflected only in the value of k. Determining $d[P]/dt$ when $n \neq 1$ will introduce no error since $[P] = 0$ at zero time. However, determining $-\,d[S]/dt$ when $n = 2$ will give the rate expression the form of eqn. 2_e or 2_f, while other values of n, except $n = 0$, will give somewhat different curves when plotted in the usual manner.

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REFERENCES

[1] M. DIXON AND E. C. WEBB, *Enzymes*, Academic Press, Inc., New York, 1958, p. 19; p. 73.
[2] R. B. MARTIN AND C. NIEMANN, *Biochim. Biophys. Acta*, 26 (1957) 634.
[3] J. P. WOLF AND C. NIEMANN, *J. Am. Chem. Soc.*, 81 (1959) 1012.
[4] J. M. REINER, *Behavior of Enzyme Systems*, Burgess Publ. Co., Minn., Minn., 1958, p. 89.
[5] J. Z. HEARON, S. A. BERNHARD, S. L. FRIESS, D. J. BOTTS AND M. F. MORALES in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, 2nd edit., Vol. I, Academic Press, Inc., New York, 1959, p. 77.
[6] K. LAIDLER, *The Chemical Kinetics of Enzyme Action*, Oxford, 1958, p. 101.
[7] R. J. FOSTER AND C. NIEMANN, *Proc. Natl. Acad. Sci. U.S.*, 39 (1953) 999.
[8] T. H. APPLEWHITE AND C. NIEMANN, *J. Am. Chem. Soc.*, 77 (1955) 4923.
[9] R. R. JENNINGS AND C. NIEMANN, *J. Am. Chem. Soc.*, 77 (1955) 5432.
[10] K. A. BOOMAN AND C. NIEMANN, *J. Am. Chem. Soc.*, 77 (1955) 5733.
[11] W. E. M. LANDS AND C. NIEMANN, *J. Am. Chem. Soc.*, 77 (1955) 6508.
[12] J. T. BRAUNHOLTZ, R. J. KERR AND C. NIEMANN, *J. Am. Chem. Soc.*, 81 (1959) 2852.

AN IMPROVED ISOLATED SINGLE ELECTROPLAX PREPARATION

I. EFFECT OF COMPOUNDS ACTING PRIMARILY AT THE SYNAPSES

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SUMMARY

Acetylcholine and related structures have been tested as to their effects on the electrical activity of an improved isolated single electroplax preparation. As a measure for comparing the relative strength of the compounds, the concentration which produced 50 % decrease in spike height within 20 min or less (usually from 5–15 min) was used. This concentration is referred to as the minimum active concentration. (a) Acetylcholine, decamethonium and *d*-tubocurarine were about 5–10 times as active as in previous preparations; no marked increase in sensitivity was observed with neostigmine and dimethylaminoethyl acetate. (b) Among the newly tested compounds succinylcholine proved to be twice as strong as acetylcholine, propionylcholine about 100 times weaker, and benzoylcholine several hundred fold weaker; the S-analogue of acetylcholine was about 3–5 times weaker. Dimethyl curare is only about half as strong as curare. (c) The decrease in spike height caused by the various quaternary compounds tested showed much better reversibility than in the previous preparation. (d) Curare in concentrations about 1000 times as high as those required to block the indirect response, abolished the propagated spike evoked by direct stimulation. In previous preparations no effect was obtained with curare outside the synaptic region even in higher concentrations than those applied in the present studies. (e) Following block of the indirect spike by curare, acetylcholine when applied in higher concentrations was able to overcome the block thus adding new evidence for the competitive action between acetylcholine and curare for the receptor. Dimethylaminoethyl acetate and the S-analogue of acetylcholine did not affect the direct spike in presence of curare in the concentrations tested. (f) Dimethylaminoethyl acetate acts much more strongly at pH 6 to 8, where it is present mostly in the cationic (protonated) form, than at pH 9, where it is present mostly in its uncharged form. The role of the Coulombic forces in the receptor protein thus appears similar to that previously observed with acetylcholinesterase. (g) The various changes of the response to acetylcholine and related structures after the removal of the adjacent membrane are a new demonstration that the effect of externally applied compounds on biological membranes depends to a great extent on the permeability barriers protecting the active membrane.

Abbreviations: DMAEA, dimethyl aminoethyl acetate; ACh, acetyl choline; S-ACH, acetoxyethyl dimethyl sulfonium sulfate; TBC, tubocurarine chloride.

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INTRODUCTION

Electric organs of electric fish have been used, since 1937, by NACHMANSOHN and his colleagues for elucidating the role of acetylcholine in the generation of bioelectric currents. The results up to date have been summarized in a recent monograph[1]. During the last decade isolated rows of electroplax of the electric eel (*Electrophorus electricus*) have been used in the laboratories of CHAGAS AND NACHMANSOHN for the analysis of electrical characteristics and their relation to the effects of acetylcholine and related compounds known to react with the acetylcholine system (see for instance ref. 2–6). However, due to the large number of cells in these preparations and the presence of many extracellular membranes surrounding the electroplax, forming strong permeability barriers for many compounds, the results were in many respects unsatisfactory. In particular, they were completely unsuitable for any investigation of ion movements associated with electrical activity and the study of chemical effects on such movements.

A new important development started with the preparation of a single isolated electroplax by SCHOFFENIELS during the years 1956 to 1958 (see ref. 7–9). A single cell is mounted in a chamber in such a way that it separates two pools of fluid, one pool bathing the innervated conducting membrane, the other the noninnervated and nonconducting membrane. Thus, both membranes can be exposed separately to chemical compounds and the effects of the exposure on ion flux and electrical activity can be examined. In the first observations of SCHOFFENIELS in which the effects of acetylcholine and related structures were studied, an extracellular membrane of the adjacent compartment was still attached to the innervated membrane[7]. Later SCHOFFENIELS improved this preparation by removing this membrane through dissection. Only a minimum amount of ground substance at most 100 μ thick but possibly less, is still present in front of the conducting membrane of the cell. SCHOFFENIELS initiated studies on ion flux with this improved preparation[8,9] and these studies were later continued in this laboratory by WHITTAM AND GUINNEBAULT[10,11].

It appeared necessary for many reasons to re-examine and re-evaluate systematically the effects of acetylcholine and related structures with the improved preparation of SCHOFFENIELS. Such study promised to provide pertinent quantitative data as to the effects of the compounds on the innervated membrane without interference of a strong extracellular structure which must have greatly modified these effects. This and the following paper present the results of these investigations.

The innervated membrane contains a large number of synapses, about 20 to 50,000 per electroplax, dependent on the size of the cell. This paper summarizes the results obtained with compounds acting primarily on the synaptic junctions. The second paper describes effects of compounds acting on both synaptic and conducting membranes.

METHODS

The procedure used for the isolation of a single electroplax was essentially the same as described previously[6–8]. A slice of tissue was cut from the caudal part of the electric eel (organ of SACHS); a single row of cells was isolated from the slice and tied in a frame and covered with oxygenated Ringers' solution. The cells were dissected under microscopic control as close as possible to the innervated membrane.

Only undamaged electroplax were used in the experiments. A single electroplax unit was mounted between two pools of Ringer's solution in a special chamber. The dissection allowed the innervated membrane of the cell to be placed firmly and directly against a window (8 mm long and 0.4 mm wide) punched out of a sheet of nylon. The chamber permits washing of the two cellular membranes by separate pools of oxygenated Ringer's solution. Compounds dissolved in one pool could pass to the other pool only through the cell. All compounds were added to the pool bathing the innervated membrane unless otherwise stated. The compounds were dissolved in Ringer's solution of the following composition in μmoles/ml: NaCl, 160; KCl, 5; $CaCl_2$, 2; Na_2HPO_4, 1.2; NaH_2PO_4, 0.3 and glucose, 10. The pH was 7.0 to 7.2 except where stated when the pH was made more alkaline or more acid by addition of NaOH or HCl.

The electrical activity was recorded with extracellular electrodes dipped in the solutions bathing the innervated and noninnervated membranes. The cell was stimulated by monophasic pulses of 0.1 msec duration and of controlled recorded intensity. The magnitude of the action potential in mV was recorded from a cathode ray oscilloscope. The conducting membrane of the electroplax was stimulated either directly by a cathodic pulse applied to the innervated membrane or indirectly by reversing the polarity of the stimulus[5].

RESULTS

Table I summarizes the compounds tested. In low concentrations they affect electrical activity by acting primarily at the synapses. The concentration required for decreasing the height of the response by 50% within 20 min or less has been taken as criterion for the strength of action of a compound rather than complete block because with some compounds very small potentials may be elicited for a long time. These are probably local, nonpropagated responses and may persist for a long time

TABLE I

MINIMAL ACTIVE CONCENTRATIONS* OF ACETYLCHOLINE AND RELATED STRUCTURES WHICH AFFECT ELECTRICAL ACTIVITY OF THE ISOLATED SINGLE ELECTROPLAX BY ACTING PRIMARILY AT THE SYNAPSES

The first six compounds listed were tested in the presence of 34 mμmoles/ml of physostigmine.

Compounds	*No. of Expts.*	*Minimal active concentration*	
		mμmoles/ml	*Time min*
Acetylcholine	7	2–4	5–10
Dimethylaminoethyl acetate	7	200–1500	5–20
Propionylcholine	3	250–500	8–18
Benzoylcholine	3	600–1200	11–16
Succinylcholine	3	1.5	8–17
β-acetoxy ethyl dimethyl sulfonium sulfate	3	12	5–10
Carbamylcholine	3	25–50	5–20
Decamethonium	4	2–4	5–10
Neostigmine	3	50–100	4–20
d-tubocurarine	6	1–7	6–16
Dimethylcurare	2	3	2–10

* Concentration required to decrease spike height by 50% within 20 min or less (see last column).

after the propagated spike potential has been abolished. With other compounds no such local responses are observed. Therefore, the complete abolition does not appear to be the best measure for comparing the strength of a compound. The difference in block of electrical activity is not surprising. Although all the compounds tested affect electrical activity by reacting with the acetylcholine system, their affinity to the various protein members of the system differs greatly[1]. Therefore, additional factors are introduced when complete block is taken as the measure of the strength of compounds not affecting electrical activity in a strictly identical and comparable manner, *i.e.*, by the reaction with the same protein and with the same relative affinity: in one case local responses may be elicited but not in another. A 50% decrease of the response within a given time has therefore been selected as an indication of comparative strength. The concentration required to produce this effect has been referred to as minimal active concentration. All compounds, with the exception of dimethylcurare, were tested on cells of two or more electric eels.

The cells showed relatively small variations in their response to most of the compounds tested. Surprisingly, however, the response to curare was not regular. The greater part of the cells responded to a few μg/ml of curare (see Table I) but some of them were resistant to concentrations as high as 100 μg/ml. These curare resistant cells responded nevertheless to other quaternary compounds, such as carbamylcholine and decamethonium, in the usual concentrations. We do not have, at present, an explanation for this irregularity in behavior towards curare.

According to theory, the acetylcholine system is essential for the generation of bioelectricity[1]. However, acetylcholine and many other closely related quaternary nitrogen compounds, when applied externally, do not affect conducting membranes of nerve and muscle fibers. The compounds are lipid insoluble and their failure to act on axonal conduction has been attributed to the presence of structural barriers preventing the compounds from reaching the active site. This viewpoint has been experimentally supported in various ways[12,13]. However, in the electroplax they abolish electrical activity and simultaneously depolarize the cell. This simultaneous block of the response to both direct and neural stimulation was first assumed to indicate the absence of a structural barrier protecting the conducting membrane of the electroplax of *Electrophorus electricus* against these types of compounds. However, *d*-tubocurarine (curare) blocks in low concentration only the indirect spike. Even in high concentrations the direct spike appeared in earlier experiments to be unaffected. When acetylcholine and other depolarizing agents were applied in presence of low concentrations of curare, the direct spike was not affected, contradicting the assumption of the absence of structural barriers. Since the conducting membrane of an electroplax contains many thousands of synapses, depolarization of so many points of the membrane produces a short circuit of the entire membrane and it is this short circuiting which is responsible for the block of the direct spike and not a direct action[6].

Curare does not depolarize. Therefore, it cannot produce a short circuiting of the conducting membrane. In low concentrations, about 1 mμ mole/ml, it blocks the indirect but not the direct spike. However, if applied in 1 μmole/ml, it has now been observed with the new improved preparation, that it does block the direct spike (Fig. 1). Apparently, when used in high concentrations, small amounts of curare are able to penetrate and these small amounts are sufficient to act directly upon the conducting membrane. The barrier is relative and not absolute.

A small amount of physostigmine was always present in the solutions containing esters which are hydrolyzed by acetylcholinesterase. The concentration of physostigmine added was about 0.1 the minimal amount necessary to affect either the direct or the indirect spike. Acetylcholinesterase is present in several fold excess. Physostigmine in the small concentrations used inhibits most likely a fraction of cholinesterase, and possibly other esterases, which permits the externally applied ester to reach the active site and to act before it is hydrolyzed. In the absence of physostigmine, about thousand times as much acetylcholine is required for blocking electrical activity, as in its presence. DMAEA, the tertiary analogue of acetylcholine, is also capable of depolarizing the synaptic junction; however, much higher concentrations are required[4].

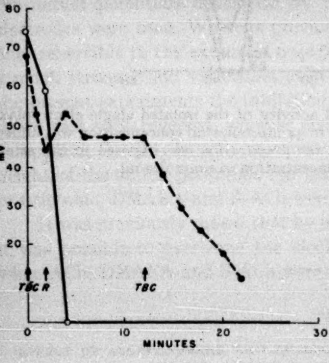

Fig. 1. Effect of TBC in high concentration on the direct response of the isolated single electroplax of *Electrophorus electricus*. The compound was added to the pool bathing the innervated membrane. The response (in mV) to direct ●---● and indirect ○—○, stimulation, was recorded by an oscilloscope using extracellular electrodes. In the first exposure 3500 mμmoles/ml of TBC were used, in the second 1400 mμmoles. At R the preparation was washed with Ringer's solution.

The potency of DMAEA did not vary significantly when applied at a pH range from 6 to 8. When, however, the pH was adjusted to 9.3, its potency fell drastically to about 15 to 25 % of that observed at pH 6–8 (Fig. 2). Tris(hydroxymethyl)aminomethane in 1.5 m*M* concentration, was used as the buffering agent in these experiments instead of phosphate buffer in order to avoid precipitation at alkaline pH. The buffer did not affect the electroplax action potential.

In the first observations with the isolated single electroplax preparation it was possible to obtain recovery of electrical activity after block by tertiary nitrogen

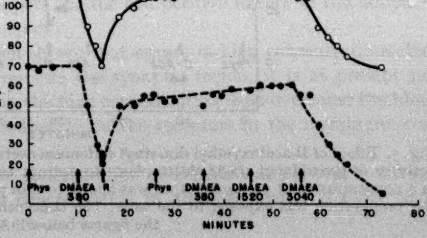

Fig. 2. Effect of DMAEA at pH 6.5 and 9.5 on the electrical activity of the isolated single electroplax. Recordings and symbols as in Fig. 1. Physostigmine (Phys), in a concentration of 34 mμmoles/ml was added 10 min prior to DMAEA and was always present when the preparation was exposed to the ester. The concentration in mμmoles/ml are indicated by the figures below DMAEA.

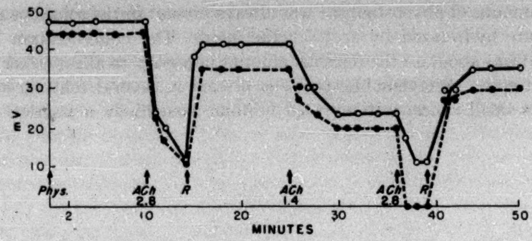

Fig. 3. Reversibility of ACh action on the electrical activity of the isolated single electroplax. Recordings and symbols as in Fig. 1. Physostigmine, in 34 mμmoles/ml concentration was added 10 min prior to ACh and was always present when the preparation was exposed to the ester. The figure below ACh indicates its concentration in mμmoles/ml.

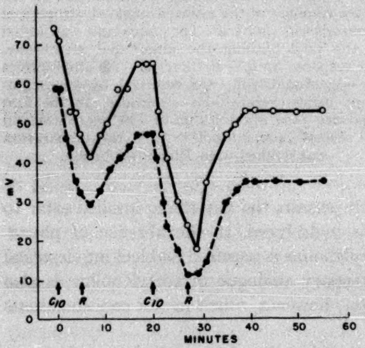

Fig. 4. Reversible effect of decamethonium iodide (C_{10}) on the electrical activity of the isolated single electroplax. Recordings and symbols as in Fig. 1. The concentration of decamethonium iodide applied was 2 mμmoles (0.5 μg)/ml.

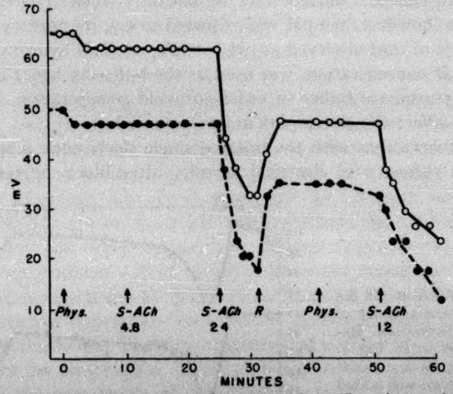

Fig. 5. Effect of B-acetoxyethyl dimethyl sulfonium acetate (S-analogue of ACh) on the electrical activity of the isolated single electroplax. Recordings and symbols as in Fig. 1. Physostigmine, in a concentration of 34 mμmoles/ml was added 10 min prior to S-ACh and always present when the preparation was exposed to the ester. The concentrations in mμmoles/ml are indicated by the figures below S-ACh.

compounds. However, recovery after block by quaternary compounds was only partial and transitory. With the improved preparation the recovery has now been found to be much more marked and lasting. Figs. 3 to 5 show results obtained on testing the reversibility of ACh, decamethonium (C_{10}) and the sulfur analogue of acetylcholine, B acetoxyethyl dimethyl sulfonium sulfate. The mV recorded are not the actual potentials developed by the cell, since external and not intracellular electrodes were used. Whereas previously the block produced by acetylcholine was only reversible to the extent of 0–40 % and sometimes only transitory, we now find a much stronger and sometimes even complete and persisting reversal of block. In the present experiments the inhibition of electrical activity by decamethonium could be at least 50 % reversed, whereas previously no reversibility was obtained with this compound. We also found a marked reversal of the effect of carbamylcholine on the height of the action potentials (50 % or more) and the results with succinyl choline, prostigmine, DMAEA and S-ACh were similar.

It was previously shown that by increasing the concentration of carbamylcholine it was possible to overcome the block by curare[6]. Table II shows results obtained when ACh, DMAEA and S-ACh were applied to the improved preparation following curare. About 50 times the minimal active concentration of acetylcholine is required to overcome block at the synapse by 7 mμmoles/ml curare, while 2000 times the minimal active concentration was required to overcome block by 350 mμ moles/ml curare. These findings add new support for the competitive nature of the action of acetylcholine and curare on the receptor.

TABLE II

EFFECT OF ACETYLCHOLINE AND ANALOGUES ON THE DIRECT RESPONSE OF THE ISOLATED SINGLE ELECTROPLAX IN THE PRESENCE OF CURARE

The minimal active concentrations of ACh, DMAEA and the S-analogue of acetylcholine were tested. Curare in the final concentrations listed was applied to the fluid bathing the innervated membrane. The indirect response was blocked by curare prior to the application of ACh, DMAEA and S-ACh. The compounds were tested in the presence of the same concentration of curare and in the presence of 0.034 μmoles/ml physostigmine.

Curare μmoles/ml	*Compound*	*Minimal active concentration μmoles/ml*	*Highest concentration tested μmoles/ml*	*Time min*
0.07	S-ACh	No effect	0.24	
0.07	DMAEA	No effect	30.0	
0.007	ACh	0.06–0.14		8–9
0.07	ACh	0.28–0.56		2
0.35	ACh	5.6		20

However, since it has now been shown that curare in high concentrations does affect the conducting membrane outside the synaptic region, it is at present not possible to ascertain whether ACh in the high concentration used overcame the block only at the synapses or acted also directly on the receptors in the membrane conducting the propagated spike.

Fifty times the minimal active concentration of DMAEA at pH 7 or 8 did not overcome curare block; since this concentration in absolute figures is quite high, higher concentrations were not tested.

DISCUSSION

The compounds tested in this paper and that which follows have been divided into two groups according to their effects on the electrical activity of the cell[1,5]. The members of one group, mainly quaternary ammonium derivatives, block electrical activity, by reacting primarily with the receptor at the synapses and simultaneously depolarize the membrane. These compounds are referred to as receptor activators. The members of the second group, referred to as receptor inhibitors, block electrical activity but do not cause depolarization. The tetrahedral structure of the quaternary nitrogen group apparently induces a change of configuration of the protein in which the protein envelops the more or less spherically shaped cationic group. The possibility of such a folding was suggested by the observation that the activities of acetylcholinesterase and choline acetylase are greatly enhanced by the presence of an extra methyl group compared to those when the tertiary analogues are used[14]. Experimental evidence supporting the assumption of a configurational change was offered by the observations on the change of entropy of activation (ΔS^*) by the hydrolysis of acetylcholinesterase: it is very unfavorable in the case of the tertiary analogue of acetylcholine and becomes very favorable in the case of acetylcholine, *i.e.*, in the presence of the extra methyl group[15]. Local changes in configuration of the receptor protein may lead to the removal of strategically located positive charges in the membrane, thereby acting as the trigger for accelerated ion flux.

The compounds discussed in this paper are receptor activators except curare and dimethyl curare, which are receptor inhibitors. The sulfur analogue of acetylcholine has not yet been tested for its ability to produce depolarization, so that it is not yet known whether it is a receptor activator or inhibitor. This compound is similar to acetylcholine in many ways, *i.e.*, it reacts in small concentrations with the receptor, it is ineffective in the usual concentration following curare, and it has a charged group. But it differs in that there are only two methyl groups on the sulfur atom compared to the three on the nitrogen in acetylcholine. It will be interesting to determine whether this compound has a depolarizing action and whether the ΔS^* of its hydrolysis by acetylcholinesterase is favorable, thus indicating a configurational change of the protein.

Previously, when rows of cells were used or single cells not as finely dissected as in the present experiments, the concentration of acetylcholine required for blocking electrical activity was about 30–60 mμmoles/ml or more. Although the measure of effective strength is not the same as that used in previous experiments, the ester acts on the improved preparation in concentrations of about 2–4 mμmoles/ml. Moreover, the reversibility of the action is now much more marked and satisfactory than previously. The method of applying acetylcholine is still extremely crude compared with the biological process, in which acetylcholine is simultaneously liberated in a period of μsec in thousands of synapses within the active membrane. Under those conditions still very much smaller amounts, probably by several orders of magnitude, must be assumed to be sufficient for the generation of bioelectricity. The crude method of applying acetylcholine and possibly the presence of thousands of synapses may make it difficult to activate simultaneously the amount of receptors required for the electrogenic action, so that only a blocking effect is observed. Synchronization may be a pre-requisite for obtaining the postsynaptic potential. Moreover, by recording the overall activity of what is occurring at synapses without synchronization it may be quite difficult to observe any positive electrogenic effect at any one synapse, since very rapidly excitation may be followed by block.

Comparing the concentrations of some of the other compounds with those previously required for block, we find decamethonium and curare to act in 5–10 times lower concentrations than those used previously. Neostigmine and DMAEA, on the other hand, acted in concentrations similar to those reported previously. Most of the other compounds have not been tested before on the electroplax. Succinylcholine is, on a molar basis, twice as potent as acetylcholine. The compound resembles two acetylcholine molecules combined. Diquaternary nitrogen derivatives in which the two quaternary nitrogens are separated by a distance of about 14 to 15 Å, are much stronger bound to acetylcholinesterase[16] and apparently to the receptor protein[17] than corresponding monoquaternary compounds. There is apparently a second negative charge in the surface of the protein at this distance from the anionic site; thereby the binding is greatly enhanced. Since each of the quaternary groups of succinylcholine should be able to produce the folding, the stronger binding by the other nitrogen readily accounts for the stronger action. The specificity of the receptor for acetylcholine appears quite remarkable when one compares the potency of propionylcholine with that of acetylcholine: one additional methyl group in the acyl decreases the potency hundred fold. This may be due to steric hindrance.

The removal of a single methyl group from the quaternary nitrogen of acetylcholine as in DMAEA decreases the potency about 200 fold. Here, however, different factors must be considered: DMAEA, although a tertiary amine, is capable of causing depolarization[5]. DMAEA has a p*K* of 8.3 and therefore it will exist at pH 6–8 from 99% to 67% in its charged form in which the methyl group of the quaternary nitrogen analogue is replaced by a proton. It appears likely that the charged form is the active one: the substitution apparently greatly weakens but does not abolish completely the activating effect on the receptor protein. This view is supported by the fact that no marked change in the potency of DMAEA was observed when the pH was changed in the range from 6 to 8. In this range the percentage of the charged form was reduced only by about one third. This decrease may be too small to produce a significant effect considering the complexity of biological material. At pH 9.3, however, the potency of DMAEA decreased by a factor of 4–8, corresponding roughly to a decrease in percentage of charged DMAEA to about one sixth of what it is at pH 8. This observation strongly supports the assumption that it is indeed the protonated cationic form of DMAEA which is the active species. The demonstration of the importance of Coulombic forces in the interaction between the small molecule and the receptor protein is of particular interest when compared with the observations on the hydrolysis of ACh and DMAEA by acetylcholinesterase[18]. The rate of hydrolysis of the tertiary analogue is lower than that of the quaternary form, but it does not differ markedly between pH 6 and 8; at pH 9, however, the rate has strongly decreased. It is interesting to note here again, as in other cases, the apparent similarity of molecular forces effective in the active surface of the proteins of the acetylcholine system.

The actual pH in the conducting membrane of the electroplax in these experiments is, of course, not known. Only the external pH of the surrounding fluid has been changed. However, since the potency of DMAEA did change markedly with the pH of the external fluid, it seems possible that the pH in the conducting membrane, at least at the poorly protected region of the synapse, may have been altered to some extent.

Using the improved preparation, it has been possible for the first time to block the direct response by the action of curare. About a thousand times higher concentration of curare is required to block the response to direct stimulation than that which blocks the response to neural stimulation. This effect serves to emphasize the point that although the conducting membrane is protected by a strong permeability barrier in contrast to the synapses there is no fundamental difference between the membrane and the synaptic areas as far as the primary role of the acetylcholine system is concerned, since curare is able to act on both sites. A strong further support are the recent findings of Dettbarn[19] that curare is capable of blocking rapidly and reversibly electrical activity at the Ranvier node.

The greater sensitivity of the preparation, the better reversibility of the effects and the action of curare on the propagated spike emphasize again the importance of permeability in evaluating effects of compounds on the intact cell.

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REFERENCES

[1] D. Nachmansohn, *Chemical and Molecular Basis of Nerve Activity*, Academic Press, New York, 1959.
[2] D. Albe-Fessard, C. Chagas and H. Martins-Ferreira, *Ann. acad. brasil. sci*, 23 (1951) 327.
[3] R. D. Keynes and H. Martins-Ferreira, *J. Physiol. (London)*, 119 (1953) 315.
[4] M. Altamirano, C. W. Coates, H. Grundfest and D. Nachmansohn, *J. Gen. Physiol.*, 37 (1953) 91.
[5] M. Altamirano, W. L. Schleyer, C. W. Coates and D. Nachmansohn, *Biochim. Biophys. Acta*, 16 (1955) 268.
[6] E. Schoffeniels and D. Nachmansohn, *Biochim. Biophys. Acta*, 26 (1957) 1.
[7] E. Schoffeniels, *Biochim. Biophys. Acta*, 26 (1957) 585.
[8] E. Schoffeniels, *Science*, 127 (1958) 1117.
[9] E. Schoffeniels, *Ann. N.Y. Acad. Sci.*, 81 (1959) 285.
[10] R. Whittam and M. Guinnebault, *J. Gen. Physiol.*, 43 (1960) 1171.
[11] R. Whittam and M. Guinnebault, *Biochim. Biophys. Acta*, in the press.
[12] T. H. Bullock, D. Nachmansohn and M. A. Rothenberg, *J. Neurophysiol.*, 9 (1946) 9.
[13] M. A. Rothenberg, D. B. Sprinson and D. Nachmansohn, *J. Neurophysiol.*, 11 (1948) 111.
[14] R. Berman, I. B. Wilson and D. Nachmansohn, *Biochim. Biophys. Acta*, 12 (1953) 315.
[15] I. B. Wilson and E. Cabib, *J. Am. Chem. Soc.*, 78 (1956) 202.
[16] F. Bergmann, I. B. Wilson and D. Nachmansohn, *Biochim. Biophys. Acta*, 6 (1950) 217.
[17] S. Ehrenpreis, *Science*, 129 (1959) 1613.
[18] F. Bergmann, I. B. Wilson and D. Nachmansohn, *J. Biol. Chem.*, 186 (1950) 693.
[19] W. D. Dettbarn, *Nature*, 186 (1960) 891.

Short Communications

Structure of the paramylon from *Euglena gracilis*

Both photosynthetic and non-photosynthetic unicellular algae of the family *Euglenaceae* contain in their cytoplasm characteristic granules of diverse form and number. GOTTLEIB[1] in 1850 named them paramylon granules since they were morphologically similar to starch but did not show a blue colour with iodine. Similar granules have been described in the filamentous green alga *Cladophora rupestris*[2]. No chemical characterisation, beyond the demonstration that paramylon is a glucose-containing polysaccharide, has been recorded[3-6]. Further details of the chemistry of the paramylon from *Euglena gracilis* are now reported.

Cultures of *E. gracilis* were grown on an inorganic salts–vitamin medium[7] containing 15 % sucrose and the granules were separated as follows. Cells were collected by centrifugation, washed twice with water, once with ethanol and the pigments extracted with chloroform–ethanol (2:1, v/v). The depigmented cells were disrupted and most of the protein removed by incubation overnight at 40° with 1 % trypsin (B.D.H., Ltd.) in 0.1 *M* phosphate buffer at pH 7.6. The residue was extracted twice with 90 % urea and washed with water. The off-white material consisting mainly of paramylon granules was further deproteinised by SEVAG's method[8]. The white suspension of paramylon granules was collected by centrifugation, washed with water and dried *in vacuo* at 60°. The yield represented 25 % of the weight of the dried depigmented cells.

Microscopic examination of material prepared in this way showed no intact cells or cell debris. The granules were refractile, homogeneously anisotropic, somewhat lenticular in shape and measured 2.3–2.8 (mean 2.6)$\mu \times$ 1.0–1.6 (mean 1.3)μ. Electron microscopy showed a membrane enclosing fibrils arranged lengthwise in the granule. Their staining behaviour corresponded to that reported in the literature[1,9] and in addition no yellow fluorescence was observed with ARNOLD's callose stain[10].

The granules were soluble in 5 % NaOH, 55 % H_2SO_4, formaldehyde and anhyd. formic acid, and were more or less swollen by lower concentrations of these reagents and by 5 % calcium thiocyanate, 25 % KI, satd. $ZnCl_2$, formamide and cuprammonium. The paramylon showed $[\alpha]_D^{16} = +26°$ in anhyd. formic acid (*c*, 2), $[\alpha]_D^{16} = +28°$ in 2.5 *N* NaOH (*c*, 2). Found: C, 42.4; O, 49.3; H, 6.49; N, 0.5; P, 0.05. $(C_6H_{10}O_5)_n$ requires C, 44.5; O, 49.4; H, 6.16 (Dr. K. W. ZIMMERMAN, C.S.I.R.O., Organic Microanalytical Laboratory, University of Melbourne).

Paramylon was completely hydrolysed by refluxing 20 mg with 2 ml 90 % formic acid on a boiling-water bath for 2 h followed by a further 3-h refluxing after the addition of 7 ml 3 *N* H_2SO_4. Paper chromatography of the neutralised hydrolysate showed a single reducing compound with the same mobility as glucose in four solvent systems. The identity of the hydrolysis product was confirmed by preparation of the 2,5-dichlorophenylhydrazone derivative (m.p., 160° (corr.); authentic glucose

2,5-dichlorophenylhydrazone, m.p. 160° (see ref. 11)). The polyglucose content of the paramylon was 98.8% after correction for the loss of glucose under the hydrolysis conditions.

A partial hydrolysate was prepared by refluxing paramylon with 90% formic acid (10 mg/ml) for 3 h on a boiling-water bath and after distillation of the formic acid the residue was dissolved in water. Chromatography in *n*-propanol–ethyl acetate–water (6:1:3, v/v) showed a series of six reducing compounds when sprayed with $AgNO_3$. The two compounds with the greatest R_F values corresponded in mobility to glucose and laminaribiose and a plot of the logarithm of the partition function against the expected number of hexose units/molecule was linear, suggesting that the reducing compounds were oligosaccharides forming a homologous series. Paper electrophoresis of the partial hydrolysate in borate buffer at pH 10 showed compounds with M_G's of 1.0, 0.69 and 0.59. The M_G of laminaribiose was 0.69. Preparative paper chromatography was used to separate the di- and tri-saccharides from a large-scale partial hydrolysate. The separated compounds were acetylated with acetic anhydride in pyridine. The disaccharide acetate had m.p. (corr.) 163–164° (β-laminaribiose-octa-O-acetate, m.p. 160–161° (see ref. 12)), the trisaccharide acetate m.p. (corr.) 122–123° (β-laminaritriose-hendeca-O-acetate, m.p. 120–121° (see ref. 12)). Both melting points were determined on a heated microscope stage.

On oxidation with sodium metaperiodate the paramylon granules as isolated and paramylon reprecipitated from NaOH solution showed a periodate consumption, measured spectrophotometrically[13], of less than 0.02 mole/mole anhydroglucose.

The infrared spectrum of the sample dispersed in KCl and examined as a pressed disc was well defined and a band of moderate intensity near 890 cm^{-1} provides evidence that the material is β-linked. The X-ray pattern of the paramylon showed relatively sharp rings with the following spacings and relative intensities: 13.6 Å (VS), 7.85 Å (mw), 6.78 Å (w), 5.35 Å (vw), 4.45 Å (m.dif.), 3.92 Å (m), 3.62 Å (mw), 3.45 Å (w), 3.00 Å (vw), 2.90 Å (w), 2.70 Å (vw), 2.65 Å (vw).

This pattern agrees closely with the pattern published by KREGER AND MEEUSE[14] although no spacing values were given. On the basis of the similarity of the X-ray patterns of paramylon from *E. viridis* and *E. geniculata*, and yeast glucan which had been boiled for 2 h with dil. HCl these authors suggested that these compounds are chemically identical. The main glycosidic linkage in yeast glucan has been shown to be β-1,3 (see ref. 15, 16). The absence of the reaction of paramylon with periodate-Schiff reagent observed by PRINGSHEIM[17], and also by SINGH[18] with the paramylon of *Trachelomonas grandis* suggests that few adjacent hydroxyls were present, which is consistent with a 1,3 glucan structure for paramylon. The identity of the partial hydrolysis products and the periodate consumption of the paramylon from *E. gracilis* together with the infrared spectrum and optical-rotation data confirms the 1,3-β-glucan nature of paramylon. Whether paramylon is the substance of the granules in all *Euglenaceae* remains to be demonstrated. Thus the birefringent granules from *Astasia sagittifera* (SKUJA)[19] are soluble in hydrocarbons and chloroform but are insoluble in ether and alcohol and not stained by osmic acid, Ruthenium red or Sudan III. Also the X-ray pattern of *Astasia ocellata* granules are quite different from those of *E. gracilis* and the *E. viridis*, *E. geniculata* mixture studied by KREGER AND MEEUSE[14].

Paramylon granules disappear from *Euglena* cells under certain physiological conditions *e.g.* during cell division or during growth in the dark[20,21]; thus paramylon can probably be classified with those 1,3-β-glucans which act as reserve substances[22,23].

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[1] J. GOTTLIEB, *Ann. Chem. Pharm.*, 75 (1850) 50.
[2] H. KYLIN, *Kgl. Fysiograf. Sällskap. Lund. Forh.*, 14 (1944) 221.
[3] J. HABERMANN, *Ann. Chem., Liebigs*, 172 (1874) 11.
[4] F. KUTSCHER, *Z. physiol. Chem., Hoppe Seyler's*, 24 (1898) 360.
[5] O. BÜTSCHLI, *Arch. Protistenk.*, 7 (1906) 197.
[6] S. H. HUTNER AND R. C. HOCKETT, cited by S. H. HUTNER AND L. PROVASOLI, in A. LWOFF, *Biochemistry and Physiology of Protozoa*, Vol. 1, Academic Press, New York, 1951, p. 47.
[7] S. H. HUTNER, M. K. BACH AND G. I. M. ROSS, *J. Protozool.*, 3 (1956) 101.
[8] M. G. SEVAG, D. B. LACKMAN AND J. SMOLENS, *J. Biol. Chem.*, 124 (1938) 425.
[9] G. KLEBS, *Untersuch. Bot. Inst. Tübingen*, 1 (1883) 233.
[10] A. ARNOLD, *Naturwissenschaften*, 43 (1956) 233.
[11] I. MANDL AND C. NEUBERG, *Arch. Biochem. Biophys.*, 35 (1952) 326.
[12] S. PEAT, W. J. WHELAN AND H. G. LAWLEY, *J. Chem. Soc.*, (1958) 727.
[13] G. O. ASPINALL AND R. J. FERRIER, *Chem. and Ind.*, (1957) 1216.
[14] D. R. KREGER AND B. J. D. MEEUSE, *Biochim. Biophys. Acta*, 9 (1952) 699.
[15] D. J. BELL AND D. H. NORTHCOTE, *J. Chem. Soc.*, (1950) 1944.
[16] S. PEAT, W. J. WHELAN AND T. E. EDWARDS, *J. Chem. Soc.*, (1958) 3862.
[17] E. G. PRINGSHEIM, *Nature*, 173 (1954) 775.
[18] K. P. SINGH, *Am. J. Botany*, 43 (1956) 274.
[19] G. DEFLANDRE, *Bull. biol. France et Belg.*, 68 (1934) 382.
[20] F. MAINX, *Arch. Protistenk.*, 60 (1927–28) 355.
[21] E. G. PRINGSHEIM, *Nova Acta Leopoldina*, 125 (1956) 1.
[22] B. A. STONE, *Nature*, 182 (1958) 687.
[23] A. R. ARCHIBALD, D. J. MANNERS AND J. F. RYLEY, *Chem. and Ind.*, (1958) 1516.

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An improved synthesis of scopoletin

The occurrence of scopoletin (6-methoxy-7-hydroxy-coumarin) in cigarette smoke[1], and inhibition of root growth by external application of scopoletin to *Avena* and *Phleum* roots[2] have pointed out the need for a feasible synthesis to produce pure scopoletin in good yield. The present communication reports an improved synthesis of scopoletin based on modifications of the method reported by AGHORAMURTHY AND SESHADRI[3]. The pure scopoletin thus prepared has been used for preliminary studies of its metabolism in laboratory rats[4].

The new, modified procedure gives an overall yield of pure scopoletin of 55 %. In our hands, the procedure of AGHORAMURTHY AND SESHADRI gave a considerably lower yield as large losses occurred in isolating the scopoletin in pure form from mixtures of isomers and other related products.

For the synthesis of scopoletin, 12.8 g $NaHCO_3$ and 13 g benzyl chloride were added to 500 ml of a solution of 25 g esculin (6-glucoside of 6,7-dihydroxycoumarin) in absolute ethanol, and the reaction mixture was refluxed 48 h on a steam bath. 1 l water was added to the cooled solution, and the resulting precipitate was filtered and dried, m.p. 182°. 30 g of the 7-benzylesculin were hydrolyzed, using 500 ml 7 % H_2SO_4, with heating on a steam bath for 6 h. The white precipitate that formed after cooling in a refrigerator was recrystallized by solution in methanol and addition of water until cloudiness resulted, and then again cooling in the refrigerator; m.p., 192°.

The 7-benzylesculetin (14.2 g) was dissolved in 300 ml anhydrous acetone, and 21.8 g anhydrous K_2CO_3 and 14.6 g dimethyl sulfate were then added, and the mixture was refluxed for 8 h on a steam bath. After filtration, the filtrate was evaporated to about 25 ml, and water added until the 7-benzyl-6-methyl ether of esculetin precipitated. After recrystallization from aq. methanol, its m.p. was 123°. The 7-benzyl-6-methyl ether of esculetin (13.8 g) was dissolved in 150 ml glacial acetic acid plus 75 ml conc. HCl and heated for 4 h on a steam bath. After the solvent was removed *in vacuo*, the precipitate was dissolved in methanol and decolorized with charcoal. The solution was allowed to evaporate slowly to produce crystals, m.p. 205°. The compound was identical with reference scopoletin on paper-chromatographic and infrared-absorption spectral comparisons.

When developed in *n*-butanol–ethanol–$(NH_4)_2CO_3$ buffer (40:11:19, v/v/v)[5], the paper chromatogram of this scopoletin preparation revealed the presence of trace amounts of two other blue-fluorescing compounds. To remove these impurities, the scopoletin was dissolved in the least possible amount of satd. aq. Na_2CO_3. The solution was then extracted several times with 100-ml portions of chloroform, and then slowly acidified with HCl. Fine white needles of scopoletin resulted. These did not contain any blue-fluorescing substances other than scopoletin when analyzed by paper chromatography in the buffer system just mentioned.

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[1] C. H. Yang, Y. Nakagawa and S. H. Wender, *J. Org. Chem.*, 23 (1958) 204.
[2] B. M. Pollock, R. H. Goodwin and Susan Greene, *Am. J. Botany*, 41 (1954) 521.
[3] K. Aghoramurthy and T. R. Seshadri, *J. Sci. Ind. Research* (*India*), 11B (1952) 411.
[4] H. D. Braymer, *Ph. D. Dissertation*, University of Oklahoma, 1960, p. 8–15.
[5] M. E. Fewster and D. A. Hall, *Nature*, 168 (1951) 78.

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Hormonal effects on glycine metabolism in rat epididymal adipose tissue

It is now acknowledged that adipose tissue is a sensitive site of direct hormonal regulation of carbohydrate and fat metabolism[1-3]. In order to adapt to the varying requirements of the organism for fat storage, the tissue is capable of rapid and reversible growth, during which synthesis (or involution) of enzymic and structural proteins is likely to occur[4]. The present communication suggests that the regulation of amino acid and protein metabolism in this tissue may well be worthy of extensive study.

The animals used were male Wistar rats weighing between 200 and 240 g. They were fed Purina laboratory chow up to the time of death unless otherwise noted. Killing was by decapitation and the epididymal fat bodies were immediately removed, weighed on a torsion balance, and incubated for 3 h at 37° in Krebs–Ringer bicarbonate buffer. The latter contained 0.5 mM [1-^{14}C]glycine, with a specific activity of 0.66 μC/μmole. After incubation, an aliquot of the medium was used for isolating and counting CO_2 as $BaCO_3$, as previously described[5]. The fat bodies were extracted three times with chloroform–methanol (2:1), dried and homogenized in 10% trichloroacetic acid with a known amount of human albumin (Fraction V) as carrier. The precipitate was separated by centrifugation, resuspended in 10% trichloroacetic acid and heated to 90° for 15 min. It was then washed twice with fresh trichloroacetic acid, suspended in ethanol and heated to 60° for 15 min. The final precipitate was washed twice with a mixture of ethanol and ether and once with ether. After drying and weighing the material was planchetted and counted in a windowless flow counter. Appropriate corrections for self-absorption were applied. All results were expressed in terms of μmoles glycine oxidized to CO_2 or incorporated into protein per 100 mg of dry fat-free adipose tissue.

On several occasions the protein precipitate was treated with ninhydrin in the presence of carrier amino acid using the method of VAN SLYKE[6]. The CO_2 evolved was collected and found free of radioactivity. After acid hydrolysis of the protein precipitate, however, ninhydrin treatment resulted in labeling of the collected CO_2, approximately 70% of the activity present in the protein prior to hydrolysis being recovered in this fashion. Analysis of the hydrolysate by paper chromatography located the radioactivity primarily in the glycine area. The specific activity of adipose-tissue protein was approximately 10 times as great as that of the protein isolated from the diaphragm of the same animals incubated under identical conditions.

As shown in Table I, the addition of glucose to the incubating medium enhanced the incorporation of glycine into protein in tissues from both fed and fasted rats, more markedly so in the latter. In the absence of glucose, insulin had no appreciable effect on the incorporation of glycine into protein of tissues from fasted or fed animals. In the presence of glucose, however, insulin clearly stimulated the incorporation of glycine into adipose-tissue protein. Epinephrine markedly depressed glycine incorporation into protein, while accelerating its oxidation to CO_2, both effects being apparent with or without glucose. Cortisol, with or without added glucose, depressed glycine incorporation into protein without altering glycine oxidation.

These hormonal effects on the incorporation of glycine into protein by rat adipose-tissue resemble in general those described under similar conditions for rat

TABLE I

EFFECT OF HORMONES AND/OR GLUCOSE ADDED *in vitro* UPON THE METABOLISM OF [1-^{14}C]GLYCINE BY RAT EPIDIDYMAL ADIPOSE TISSUE

Hormones added in vitro	*No. of expts.*	*Glucose added (16 mM)*	*µmoles [1-^{14}C]glycine carbon per 100 mg dry fat-free tissue incorporated into*					
			Protein			*CO$_2$*		
			Control	*Hormone effect ± S.E.*	*P*	*Control*	*Hormone effect ± S.E.*	*P*
Rats fed *ad lib*								
1 None*	22*	*	0.798*	+ 0.143 ± 0.037*	< 0.001*	0.152*	+ 0.009 ± 0.011*	> 0.4*
2 Insulin 0.1 unit/ml	6	0	0.742	— 0.119 ± 0.106	> 0.3	0.117	+ 0.016 ± 0.001	< 0.001
3 Insulin 0.1 unit/ml	6	+	0.806	+ 0.333 ± 0.051	< 0.01	0.109	+ 0.047 ± 0.011	< 0.02
4 Epinephrine 18 µg/ml	6	0	0.769	— 0.593 ± 0.061	< 0.001	0.209	+ 0.407 ± 0.081	< 0.01
5 Epinephrine 18 µg/ml	6	+	0.924	— 0.533 ± 0.067	< 0.001	0.185	+ 0.521 ± 0.074	< 0.01
6 Cortisol 30 µg/ml	12	0	0.746	— 0.119 ± 0.037	< 0.01	0.125	0 ± 0.017	—
7 Cortisol 30 µg/ml	6	+	0.802	— 0.154 ± 0.057	< 0.02	0.166	— 0.017 ± 0.026	> 0.5
Rats fasted 48 h**								
8 None*	6*	*	0.569*	+ p.305 ± 0.058*	< 0.01*	0.093*	— 0.008 ± 0.025*	> 0.7*
9 Insulin 0.1 unit/ml	6	0	0.569	+ 0.028 ± 0.036	> 0.4	0.093	— 0.015 ± 0.011	> 0.3
10 Insulin 0.1 unit/ml	6	+	0.874	+ 0.241 ± 0.077	< 0.05	0.101	+ 0.012 ± 0.010	> 0.3

* Addition of glucose is the only variable, *i.e.* hormone effect = glucose effect.
** Results obtained simultaneously in tissue samples from the same 6 rats.

hemidiaphragm with, however, two notable exceptions. WOOL *et al.*[7] have reported an effect of insulin upon histidine incorporation into protein in hemidiaphragm from fasted (not from fed) rats, in the total absence of glucose, while MANCHESTER[8] observed this effect in tissues from both fasted and fed animals. Also, cortisol added *in vitro* was without effect upon glycine incorporation into diaphragm protein[9]. In rat epididymal adipose tissue insulin accelerates the incorporation of pyruvate into tissue protein even in the absence of glucose[10], a discrepancy which might derive from better ability of this substrate to support oxidation and energy production than is the case for glycine itself. Indeed, KRAHL (personal communication) has observed that pyruvate, as well as glucose, restores the insulin response for glycine incorporation into protein by this tissue.

These studies clearly suggest that the metabolism of glycine in adipose tissue is both hormonally controlled and modified by the availability of metabolic substrates such as glucose.

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[1] B. SHAPIRO AND E. WERTHEIMER, *Metabolism, Clin. and Exptl.*, 5 (1956).
[2] D. S. FREDRICKSON AND R. S. GORDON, *Physiol. Revs.*, 38 (1958) 585.
[3] A. E. RENOLD, A. I. WINEGRAD, B. JEANRENAUD AND D. B. MARTIN, *Symposium on Insulin Action*, Blackwell Scientific Publications, Ltd., Oxford, 1960, in the press.
[4] F. X. HAUSBERGER, *Diabetes*, 7 (1958) 211.
[5] A. I. WINEGRAD AND A. E. RENOLD, *J. Biol. Chem.*, 233 (1958) 267.
[6] D. D. VAN SLYKE, D. A. MACFAYDEN AND P. HAMILTON, *J. Biol. Chem.*, 141 (1941) 671.
[7] I. E. WOOL AND M. E. KRAHL, *Am. J. Physiol.*, 197 (1959) 367.
[8] K. L. MANCHESTER, *Nature*, 185 (1960) 907.
[9] K. L. MANCHESTER AND F. G. YOUNG, *J. Endocrinol.*, 18 (1959) 381.
[10] M. E. KRAHL, *Biochim. Biophys. Acta*, 35 (1959) 556.

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Isolation of cytidine 5′-monophosphate-bound carboxyl-activated peptides from *Saccharomyces cerevisiae*

After the first reports from this laboratory about the occurrence of nucleotide-bound carboxyl-activated peptides in yeast[1,2] a number of these compounds have been isolated from different organisms, and been identified in some detail[3–14]. Some authors reported the occurrence of nucleotide-peptide compounds without giving sufficient details to conclude if those compounds were carboxyl activated[15–17].

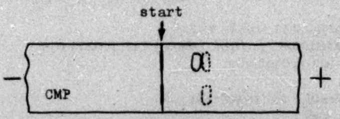
During further investigations with the aid of ion-exchange chromatography, a new carboxyl-activated nucleotide-peptide compound has been isolated from yeast.

Abbreviations: CMP, cytidine monophosphate; AMP, adenosine monophosphate.

Pressed baker's yeast was frozen in ether–CO_2, thawed, and dialysed at 3° against distilled water for about 40 h (2 replacements of the water). The pH of the dialysate was brought to about 7 by adding NH_4OH, and the solution was applied to a column of Dowex-2-Cl′-X4, 20–50 mesh, 20 cm × 4.5 cm^2. The elution was started with 0.003 *N* HCl after washing with water until the absorbancy at 260 mμ was low. The eluates were analysed by measuring the absorbancy at 260 and 280 mμ. The first component eluted was freeze-dried, and subjected twice to paper electrophoresis (0.05 *M* ammonium formate, pH 3.5, 25 V/cm, 16–20 h, 3°, Whatman No. 3 MM). A spot moving slowly in the direction of the cathode showed blue fluorescence in u.v. light (360 mμ) and a significant absorption at 260 mμ; it yielded a ferric hydroxamate colour after spraying with NH_2OH and $FeCl_3$ (see ref. 2). It was eluted, the eluate concentrated by freeze-drying, incubated with 2 *M* salt-free NH_2OH (5 min, 60°; see ref. 2) and freeze-dried. The reaction products were subjected to another fractionation by paper electrophoresis. The result of this purification is shown in Fig. 1.

A similar result was obtained after hydrolysis of the activated compound at pH 10.5 (NH_4OH, 10–15 min at 100°). After this treatment, however, spot No. 1 contained peptides instead of peptide hydroxamates.

The hydroxamates from spot No. 1 were eluted and separated by descending paper chromatography in *n*-butanol–acetic acid–water (9:1:1) at 21°. Five spots could be detected by spraying with $FeCl_3$, the approximate R_F values being 0.11, 0.16 (very weak), 0.37 (weak), 0.50 (strong), 0.60 (very weak). The spot with R_F 0.50 (ninhydrin-negative) was eluted, one half being kept as a blank, the other half being



Spot No. 1 (full lines): blue fluorescence in u.v.; hydroxamate colour after spraying with $FeCl_3$. Spot No. 2 (dotted lines): absorption in u.v.

Fig. 1. Paper electrophoresis of the reaction product of the purified carboxyl-activated compound with NH_2OH (0.02 *M* ammonium formate, pH 3.5, 15 V/cm, 20 h, Whatman No. 1).

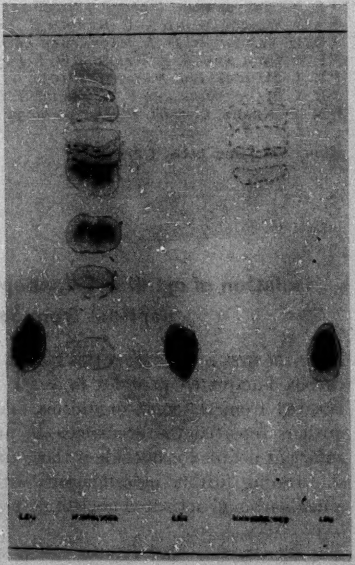


Fig. 2. Ninhydrin-sprayed chromatogram of hydrolysed and non-hydrolysed peptide hydroxamates. ——, ninhydrin positive; – – – –, weak fluorescence in u.v. light.

hydrolysed with 6 *N* HCl. Hydrolysate and blank were rechromatographed in the same solvent system. The result is shown in Fig. 2.

Spot No. 2 was analysed by measuring the u.v.-absorption spectra at pH 2.5 and 7.0, by paper chromatography in the systems isobutyric acid–conc. NH_4OH–H_2O (66:1:33; see ref. 18) and ethanol–ammonium acetate pH 7.5, (75:30; see ref. 18), and by paper electrophoresis. It appeared to be cytidine 5′-monophosphate.

Using a comparable isolation procedure, but starting from extracts of *Polyporus squamosus*, Bergkvist[17] demonstrated the occurrence of a CMP-peptide, which, however, on hydrolysis would yield cytidine 2′- and 3′-monophosphates. He did not mention results of any test for hydroxamate formation, but compared his compound with those detected by Potter and Dounce[19] in alkaline digests of ribonucleic acids.

A phosphoamide bond was proposed to account for the alkaline stability of the compounds. The cytidine-5′-monophosphate-bound peptide, however, is alkali-labile and hydroxamate positive.

The latter compound is a peptide bound through its carboxyl to CMP; the carboxyl is activated (*e.g.* it reacts easily with NH_2OH); the position of the carboxyl on the CMP moiety has not yet been established.

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[1] V. V. Koningsberger, Chr. O. van der Grinten and J. Th. G. Overbeek, *Koninkl. Ned. Akad. Wetenschap. Proc. Ser. B*, 60 (1957) 144.
[2] V. V. Koningsberger, Chr. O. van der Grinten and J. Th. G. Overbeek, *Biochim. Biophys. Acta*, 26 (1957) 483.
[3] Chr. O. van der Grinten, A. H. W. M. Schuurs and V. V. Koningsberger, *4th Intern. Congress Biochem. Vienna*, Abstracts, 1958, p. 30.
[4] J. H. Weil, G. Dirheimer and J. P. Ebel, *4th Intern. Congress Biochem. Vienna*, Abstracts, 1958, p. 21.
[5] G. Dirheimer, J. H. Weil and J. P. Ebel, *Compt. rend.*, 246 (1958) 3384.
[6] G. Harris, J. W. Davies and R. Parsons, *Nature*, 182 (1958) 1565.
[7] Chr. O. van der Grinten, *Thesis*, Utrecht, (1959).
[8] G. Harris and J. W. Davies, *Nature*, 184 (1959) 788.
[9] E. Hase, S. Mihara, H. Otsuka and H. Tamiya, *Biochim. Biophys. Acta*, 32 (1959) 298; *Arch. Biochem. Biophys.*, 83 (1959) 170.
[10] V. V. Koningsberger, *10. Colloquium d. Gesellschaft f. physiol. Chem.*, Mosbach, Springer Verlag, Berlin 1959, p. 50.
[11] J. P. Ebel, *10. Colloquium d. Gesellschaft f. physiol. Chem.*, Mosbach. Springer Verlag, Berlin 1959, p. 70.
[12] P. Mandel, *10. Colloquium d. Gesellschaft f. physiol. Chem.*, Mosbach. Springer Verlag, Berlin 1959, p. 71.
[13] P. Szafrański, E. Sutkowski and T. Gotaszeswki, *Nature*, 184 (1959) 1940.
[14] S. R. de Kloet, A. H. W. M. Schuurs, V. V. Koningsberger and J. Th. G. Overbeek, *Koninkl. Ned. Akad. Wetenschap. Proc. Ser. B*, 63 (1960) 374.
[15] D. A. Gilbert and E. W. Yemm, *Nature*, 182 (1958) 1745.
[16] A. D. Brown, *Biochim. Biophys. Acta*, 30 (1958) 447.
[17] R. Bergkvist, *Acta Chem. Scand.*, 12 (1958) 364.
[18] R. Bergkvist, *Acta Chem. Scand.*, 10 (1956) 1303.
[19] J. L. Potter and A. L. Dounce, *J. Am. Chem. Soc.*, 78 (1956) 3078.

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Incorporation of ^{32}P *in vivo* into phosphatidopeptides of rat brain

Some of the inositol phosphatides of brain occur bound to protein as phosphatidopeptides[1], and in work recently reviewed[2], these substances have been shown to incorporate ^{32}P at a relatively high rate. Subsequent reports have described the incorporation of labeled inositol into phosphatidopeptides and other lipids[3] and the incorporation of ^{32}P into phosphatidopeptide fractions in tissues other than brain[4,5]. In kidney, inositol monophosphate was demonstrated chromatographically to be a constituent of phosphatidopeptides[4]. In liver, the ^{32}P taken up into the phosphatidopeptide preparation was present in a substance which yielded glycerylphosphorylinositol and inositol monophosphate on mild alkaline hydrolysis, and was presumably similar to phosphatidylinositol[5]. In view of these findings and the fact that a major part of the phosphoinositide of brain contains inositol diphosphate[6], with phosphatidylinositol accounting for only a small proportion of the combined inositol, we have investigated the nature of the labeled phosphate compound which is obtained from rat brain phosphatidopeptides after P incorporation *in vivo*. We have found that this compound is an inositol phosphate, the chromatographic behavior of which differs from that of inositol monophosphate but is similar to that of the compound obtained from pure brain diphosphoinositide.

Adult, white, Wistar rats, weighing 300–500 g were injected with about 1 μC ^{32}P/g body weight, as unbuffered neutral sodium phosphate solution in a total volume of about 0.2 ml. After 3 h, the rats were sacrificed by decapitation, the brains removed immediately, and the phosphatidopeptides prepared by the method previously reported[1], except that the protein residue was dried by lyophilization rather than with acetone just prior to the final extraction with acidified solvent. In three separate experiments, brains of 3 to 6 rats were pooled for each extraction. It should be emphasized that this procedure does not involve a preliminary extraction of the tissue with cold 10% trichloroacetic acid, a step included in most other work on phosphatidopeptides which has thus far been reported. Because of the acidity of trichloroacetic acid, its use before extraction of lipids by neutral solvents could alter the nature of lipid–protein complexes subsequently extracted. We are currently investigating this possibility.

The phosphatidopeptide extracts were dried and the resulting solids hydrolyzed in 1 *N* alkali at 37° for 16 h[8]. The resulting hydrolysate, after acidification and treatment with trichloroacetic acid, was exhaustively extracted with ether to remove trichloroacetic acid and fatty acids, and the residual ether was removed from the water phase by warming. Soluble phosphates were prepared from this solution by precipitation with lead and decomposition of the lead precipitate with H_2S (see ref. 6), and were chromatographed on paper using the solvent of SCHORMÜLLER AND WÜRDIG[9]. The papers were sprayed with the molybdate reagent of HANES AND ISHERWOOD[10] and exposed briefly to u.v. light to make the phosphate spots visible.

After development and identification, the spots were cut out, eluted with distilled water, and transferred to aluminum planchets for counting in a Robinson gas flow counter[11]. After counting, the residue on the planchet was washed off with distilled water and total P determined[1].

Only one radioactive spot was found on the chromatograms prepared from phosphatidopeptides as described above. This spot was blue and moved to a different position than did inositol monophosphate*, sodium glycerophosphate and inorganic phosphate standards alone or mixed with the unknown. Chromatography (solvent: 90 % aq. acetone; color development with $AgNO_3$ (see ref. 12) of a deionized hydrolysate (6 N HCl, 109°, 40 h) of the radioactive material yielded only one spot corresponding to inositol.

To obtain further information about the nature of the radioactive phosphate compounds obtained in this manner from rat brain, similar but non-radioactive preparations were made on a larger scale from beef-brain white matter. For comparison, a soluble phosphate preparation was made from beef-brain potassium diphosphoinositide[6] by identical alkaline hydrolysis and lead-precipitation procedures. The phosphate solutions thus prepared were run on the same chromatogram, both separately and in mixture with standard solutions of sodium glycerophosphate and inositol monophosphate. The inositol phosphates prepared from potassium diphosphoinositide and from phosphatidopeptides behaved identically in the system used and were clearly distinct from inositol monophosphate or glycerophosphate. The diphosphoinositide also showed a small amount of inorganic phosphate which in this system is not well separated from glycerophosphate but appears yellow rather than blue. The molar ratio of P to inositol in such preparations varied from somewhat over 2 to somewhat over 3.

Thus it seems that the incorporation of ^{32}P *in vivo* into rat-brain phosphatidopeptides occurs in an inositol phosphate compound which is more highly phosphorylated than monophosphate. The presence of either the di- or triphosphate, or of a mixture of the two, would be consistent with the data, provided these two substances are indistinguishable in the chromatographic system used. We feel that the single spot found cannot be a glycerylphosphoryl diester of inositol since no glycerol could be demonstrated in the chromatograms of the acid hydrolysis products. While it is true that some loss of glycerol could have occurred by evaporation during preparation of the sample for chromatography, every attempt was made to minimize such losses. Had glycerol been present in an equimolar ratio to inositol, it would have been demonstrable on the chromatogram.

Preliminary experiments, similar to those described above, had shown that the specific activity of the P in the phosphatidopeptide fraction was about ten times as great as that of the P in the washed total lipid extract, *i.e.* the extract obtained in the same experiment by homogenizing the tissue directly with $CHCl_3$–CH_3OH (2:1). This finding was confirmed in subsequent experiments in which the lipid extracts were washed[13] and dialyzed until they were essentially free of contaminating radioactivity, and aliquots were taken for the determination of total P and for counting. The data in Table I show that the incorporation of ^{32}P into the P of phosphoinositide bound to proteins in phosphatidopeptides is about 15 times as high as the incorporation into total phospholipid-P. ANSELL *et al.*[14,15] have reported that after a 3-h equilibration period with ^{32}P *in vivo*, brain lipid inositide-P also had a specific activity about 15 times as high as total phospholipid-P. Although these workers used young rats, their data suggest the possibility that the P metabolism of phosphatidopeptides may be related to that of brain-lipid phosphoinositide. Assuming that any changes in the composition of the lipid extract occasioned by the differences between our

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* Purchased from California Corporation for Biochemical Research, Los Angeles, Calif. (U.S.A.)

TABLE I

SPECIFIC ACTIVITIES OF BRAIN PHOSPHATIDOPEPTIDES AND TOTAL PHOSPHOLIPIDS 3 H AFTER INJECTION OF ^{32}P INTO ADULT RATS

Expt.	*Specific activity**		*Ratio*
	Total phospholipid-P (A)	*Inositol phosphate-P from phosphatidopeptides (B)*	$\frac{B}{A}$
1	—	60.7	—
2	6.8	89.3	13.1
3	3.0	53.4	17.8

* Specific activities are expressed as $\frac{\text{counts/min}/\mu\text{g P}}{10^6\ \text{counts/min injected/g rat}}$

preparation methods and those used by ANSELL *et al.* did not substantially affect the radioactivity data, it would appear that the P of the phosphatidopeptide has approximately the same specific activity as the P of the lipid inositide and may originate from a common pool.

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Addendum (*received June 26th, 1960*): Since this paper was submitted, DITTMER AND DAWSON[16] have reported isolation of inositol triphosphate from a fraction obtained by steps similar to those used in our studies to prepare phosphatidopeptides.

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[1] F. N. LEBARON AND E. E. ROTHLEDER, in G. POPJÁK, *Biochemistry of Lipids*, Vol. 5, Pergamon Press, Ltd., Oxford, 1960, p. 1.
[2] J. FOLCH AND F. N. LEBARON, *Proc. Fourth Internat. Congr. Biochem.*, Vol. III, Pergamon Press, Oxford, 1959, p. 157.
[3] B. W. AGRANOFF, in R. O. BRADY AND D. B. TOWER, *The Neurochemistry of Nucleotides and Amino Acids*, John Wiley & Son, N.Y., 1960, p. 38.
[4] C. G. HUGGINS AND D. V. COHN, *J. Biol. Chem.*, 234 (1959) 257.
[5] U. FRIEDEL AND H. HERKEN, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. u. Pharmakol.*, 237 (1960) 507.
[6] J. FOLCH, *J. Biol. Chem.*, 177 (1949) 505.
[7] L. HÖRHAMMER, H. WAGNER AND J. HÖLZL, *Biochem. Z.*, 332 (1960) 269.
[8] G. SCHMIDT AND S. J. THANNHAUSER, *J. Biol. Chem.*, 161 (1945) 83.
[9] J. SCHORMÜLLER AND G. WÜRDIG, *Z. Lebensm.-Untersuch. u.-Forsch.*, 105 (1957) 397.
[10] C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
[11] C. V. ROBINSON, *Science*, 112 (1950) 198.
[12] W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 166 (1950) 444.
[13] J. FOLCH, M. B. LEES AND G. H. SLOANE STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
[14] G. B. ANSELL AND H. DOHMEN, *J. Neurochem.*, 2 (1957) 1.
[15] G. B. ANSELL AND S. SPANNER, *J. Neurochem.*, 4 (1959) 325.
[16] J. C. DITTMER AND R. M. C. DAWSON, *Biochim. Biophys. Acta*, 40 (1960) 379.

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Le carbamyl-phosphate, intermédiaire dans la dégradation de la créatinine par des extraits enzymatiques d'*Eubacterium sarcosinogenum*

L'importance biologique du carbamyl-phosphate, en tant que donateur du groupement carbamyl, a été mise en évidence chez les bactéries[1] et chez les animaux supérieurs[2]. Les résultats obtenus se rapportant principalement au rôle du CAP dans le métabolisme de la citrulline.

$$\text{Carbamate} + \text{ATP} \rightleftharpoons \text{CAP} + \text{ADP} \qquad (1)$$

$$\text{CAP} + \text{Ornithine} \rightleftharpoons \text{Citrulline} + \text{Pm} \qquad (2)$$

Toutefois, il existe une différence importante dans le mécanisme de ces réactions: réversibles chez les bactéries, elles sont irréversibles chez les animaux supérieurs[2]. C'est la réversibilité des réactions (1) et (2) dans les systèmes bactériens qui rend le CAP doublement important en tant qu'intermédiaire, puisqu'il peut servir non seulement comme donateur du groupement carbamyl, mais peut également, en présence d'ADP, conduire à la formation d'ATP.

Il sera montré, dans le présent travail, que le CAP est un intermédiaire dans la dégradation de la créatinine par des extraits acellulaires d'*Eubacterium sarcosinogenum*, Ls*.

Le milieu de culture et la préparation des extraits acellulaires ont été décrits antérieurement[3].

Il a été montré précédemment[3] que la dégradation anaérobie de la créatinine par *Eubact. sarcosinogenum* Ls. en sarcosine, NH_3 et CO_2 est associée à l'esterification de l'orthophosphate. Les expériences dans lesquelles on a utilisé [^{32}P]orthophosphate ont permis de mettre en évidence l'incorporation d'une partie de ce phosphate dans les polyphosphates minéraux[3]. Il a été également démontre[6] que l'incubation d'un extrait enzymatique de ces bactéries, en présence de créatinine, d'ADP et de [^{32}P]-orthophosphate, conduit à la formation de [^{32}P]ATP. Cette formation est liée à la dégradation de la créatinine, mais le mécanisme de ces réactions était resté obscur.

Au cours d'une étude récente, nous avons observé la présence, dans les extraits de *Eubact. sarcosinogenum* Ls d'une carbamyl-phosphate synthétase et d'une ornithine transcarbamylase, enzymes catalysant respectivement les réactions (1) et (2). Les activités de ces deux enzymes ont été mesurées par la formation de la citrulline en employant comme donateur, soit le carbamate + ATP (1), soit le CAP (2), l'ornithine étant l'accepteur du groupe carbamyl.

A la suite de ces observations une expérience a été effectuée dans laquelle la source de carbamyl a été remplacée par la créatinine. Le Tableau I montre la formation de citrulline dans ces conditions. Le CAP transféré sur l'ornithine pour former la citrulline ne peut alors provenir que de la créatinine. Le Tableau I montre également que le rapport de NH_3 formé (soit sous forme libre, soit sous forme de carbamyl fixé sur l'ornithine) sur créatinine disparue, est ici inférieur à 2, alors qu'il est sensiblement égal à 2 avec des extraits dialysés. Ceci suggère que d'autres accepteurs d'NH_3 ou du groupe carbamyl peuvent être présents dans les extraits non dialysés. Si au

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Abbréviations: CAP, carbamyl-phosphate; ADP, ATP, adénosine diphosphate et triphosphate; Pm,; Tris, tris(hydroxy)méthylaminométhane.

[*] La bactérie anaérobie stricte provisoirement désignée "*Clostridium* Ls" dans des publication antérieures[3,4] a été récemment déterminée[5] et appelée: *Eubacterium sarcosinogenum nov. sp.* ≠ Ls.

TABLEAU I

MISE EN ÉVIDENCE DE LA FORMATION DE CARBAMYL-PHOSPHATE AU COURS DE LA DÉGRADATION DE LA CRÉATININE PAR LES EXTRAITS D'*Eubacterium sarcosinogenum* LS

Le système complet contient: 0.3 ml d'extrait non dialysé (5.3 mg protéines); 10 µmoles de créatinine; 100 µmoles de tampon Tris, pH 8.5; 10 µmoles de $MgCl_2$; 10 µmoles d'ornithine; 5 µmoles d'ATP (ou d'ADP) dans un volume final de 1 ml. Incubation de 60 min à 31°. Réaction arrêtée par addition de 0.1 ml acide trichloroacétique 40%. Les dosages sont effectués sur le surnageant après centrifugation (créatinine, créatine et NH_3 sont dosés par les méthodes indiquées antérieurement[3]) (la citrulline est dosée par la méthode d'ARCHIBALD, modifiée[7]).

	Créatinine disparue (µmoles)	*NH_3 formé (µmoles)*	*Citrulline formée (µmoles)*
Système complet	5.54	5.6	3.21
Sans créatinine	0	0	0.15
Sans ornithine	3.46	6.50	0.09
Sans enzyme	0	0	0
Système complet à temps 0	0	0	0

* Les quantités de créatinine transformée en créatine ont été déduites de ces chiffres.

lieu d'ornithine on ajoute de l'acide aspartique au mélange réactionnel, il y a formation d'acide ureido-succinique que nous avons pu mettre en évidence par chromatographie sur papier.

Dans d'autres expériences effectueés dans des conditions identiques à celles résumées dans le Tableau I, mais dans lesquelles on a employé la [2-^{14}C]créatinine (marquée dans le carbone guanidique), 40 à 60 % de la radioactivité a été trouvée dans la citrulline avec une activité spécifique très voisine de celle de la créatinine au départ.

Bien que ces résultats montrent que le CAP est formé à partir de la créatinine, ils ne permettent cependant pas d'affirmer qu'il est un intermédiaire dans la dégra-

TABLEAU II

INCORPORATION DU CARBONE 2-^{14}C DE LA CRÉATININE DANS LE CARBAMYL-PHOSPHATE EN ABSENCE ET EN PRÉSENCE DE CO_2 EN EXCÈS

Le système complet contient: 0.1 ml d'extrait enzymatique (2.1 mg protéine dans l'Exp. A et 3 mg dans l'Exp. B); 20 µmoles K_2HPO_4; 50 µmoles tampon Tris, pH 8.5; 6 µmoles de [2-^{14}C]-créatinine (marquée dans le carbone guanidique); A.S. = 13,100 coups/min; 5 µmoles carbamyl-phosphate (sel de Lithium) (SIGMA); 10 µmoles $MgCl_2$. Dans l'Exp. B un échantillon parallèle contenait en plus 40 µmoles $NaHCO_3$. Volume final: 0.5 ml. Incubation 60 min à 31°. Le CAP a été séparé du mélange réactionnel par chromatographie sur papier (solvant: isobutanol–isopropanol–NH_3–H_2O (40:20:1:39). R_F respectifs: CAP, 0.26; créatinine, 0.55; créatine, 0.39. Les éluats des taches correspondant au CAP ont été concentrés par lyophylisation et leurs radioactivités déterminées. Dans l'Exp. A, le $^{14}CO_2$, provenant du carbamyl-phosphate, a été obtenu par la décomposition de ce dernier dans les cellules de Conway, à l'aide de H_2SO_4 5 *N*, puis absorbé dans la soude. Le Na_2CO_3 a été ensuite transformé en $BaCO_3$. La radioactivité de ce dernier composé a été déterminé en tenant compte de l'absorption propre du $BaCO_3$.

	Créatinine disparue (µmoles)		*2-^{14}C de la créatinine incorporé dans le CAP (coups/min)*				*2-^{14}C sous forme de $^{14}CO_2$ (coups/min)*
			Sans CO_2		*Plus CO_2*		
Expériences	*A*	*B*	*A*	*B*	*A*	*B*	*A*
Système complet	6,0	3.7	8525	3608	—	3058	5700
Système complet avec enzyme inactivée	0	0	0	125	—	209	0

dation de la créatinine. En effet, on pouvait admettre que le CAP provient exclusivement d'une synthèse *de novo* à partir de CO_2 et de NH_3 (produits finaux de la fermentation de la créatinine) par l'action de la CAP-synthétase. Pour montrer qu'il est également un intermédiaire nous avons effectué une expérience dans laquelle la [2-^{14}C]créatinine a été incubée avec CAP en absence de CO_2 ou en présence de CO_2 en excès, dans les conditions indiquées dans le Tableau II (en absence de nucléotides). Ce Tableau montre qu'une partie de la radioactivité est retrouvée dans le CAP isolé du mélange réactionnel. Le fait que la présence d'un excès de CO_2 dans la réaction ne change pratiquement pas la radioactivité incorporée dans le CAP (Tableau II, Expt. B) prouve que cette incorporation n'est pas due à un échange entre le CO_2 provenant de la créatinine et le CAP ajouté. Un tel échange aurait pû être catalysé par des traces d'ATP présentes dans l'extrait. Le Tableau II montre également que la radioactivité du CAP se retrouve, après sa décomposition chimique, dans le CO_2 qui en résulte. La diminution de la radioactivité observée dans le CO_2 ainsi obtenu est sans doute due à la décomposition partielle, au cours des multiples opérations, du CAP, composé d'une stabilité relative.

Ces résultats permettent de conclure que le CAP est un intermédiaire dans la dégradation de la créatinine, telle qu'elle est catalysée par des extraits acellulaires d'*Eubact. sarcosinogenum* Ls. Ces extraits peuvent, par ailleurs, catalyser, en présence de creatinine et d'ATP (Tableau I), la synthèse *de novo* de CAP et son transfert sur différents accepteurs. Le mécanisme enzymatique de la formation du CAP, ainsi que son rôle dans le métabolisme de la créatinine, seront décrits ultérieurement.

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1 M. E. JONES, L. SPECTOR ET F. LIPMANN, *J. Am. Chem. Soc.*, 77 (1955) 819.
2 R. L. METZENBERG, L. M. HALL, M. MARSHALL ET P. P. COHEN, *J. Biol. Chem.*, 229 (1957) 1019
3 J. SZULMAJSTER ET R. GARDINER, *Biochim. Biophys. Acta*, 39 (1960) 165.
4 J. SZULMAJSTER, *Compt. rend.*, 249 (1960) 1962.
5 J. SZULMAJSTER ET P. KAISER, *Ann. uinst. Pasteur*, 98 (1960) 774.
6 J. SZULMAJSTER, expériences non publiées.
7 S. RATNER dans S. P. COLOWICK ET N. O. KAPLAN, *Methods in Enzymology*, Acad. Press, New York, Vol. II, 1955, p. 359.

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Inhibition of enzymes by Atebrin

Inhibition of an enzymic reaction by Atebrin is frequently interpreted in the biochemical literature as a proof that a flavoprotein is involved in the reaction, especially when the inhibition is reversed by FMN or FAD. It is assumed that the drug competes with the flavin for the apo-enzyme.

It is the purpose of the present communication to draw attention to the fact,

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Abbreviations: FMN, flavin mononucleotide; FAD, flavin-adenine dinucleotide; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane.

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which has already been pointed out by others[1,2], that Atebrin is not a specific inhibitor of flavoproteins. It is shown that reversal of inhibition by FMN is not due to competition between the flavine and Atebrin for the enzyme, but is due to the formation of a compound between the Atebrin and the FMN.

Erythrocyte glucose 6-phosphate dehydrogenase and liver esterase, which are almost certainly not flavoproteins, were found to be inhibited by Atebrin.

Glucose 6-phosphate dehydrogenase activity could not be measured in the usual way, by following directly the reduction of TPN at 340 mμ, since Atebrin absorbs strongly at this wavelength. This difficulty was overcome by precipitating the Atebrin at various times by adding NaOH to pH 10. The absorbancy at 340 mμ was then measured on the clear supernatant. Recovery tests showed that no loss of TPNH occurred in this procedure. Fig. 1 shows that 5 m*M* Atebrin caused considerable inhibition of this enzyme.

Ali-esterase was studied either as a rat-liver microsomal preparation (fraction between 15,000 × *g* for 10 min and 105,000 × *g* for 60 min), or as a soluble preparation obtained by treating an acetone powder of the microsomal pellets with butanol, followed by extraction of the powder with 0.25 *M* Tris–HCl buffer, pH 7.75 (*cf.* ref. 3). The enzyme activity was determined manometrically in $NaHCO_3$–H_2CO_3 buffer. The degree of inhibition of the enzyme in microsomes at various concentrations of Atebrin is shown in Fig. 2. 50 % inhibition was obtained with 5 m*M* Atebrin, which is similar to the concentration giving 50 % inhibition of succinic oxidase in Löw's experiments[4]. The soluble ali-esterase was also inhibited to the same extent.

The ali-esterase was not inhibited by 2 μg/ml antimycin, 4 m*M* 2,4-dinitrophenol, 5 m*M* aureomycin, 5 m*M* chloropromazine, or 5 m*M* methylene blue. Chloramphenicol (5 m*M*) was slightly inhibitory. Pentachlorophenol inhibited quite strongly (50 % inhibition by 1.3 m*M*, 100 % by 5.2 m*M*).

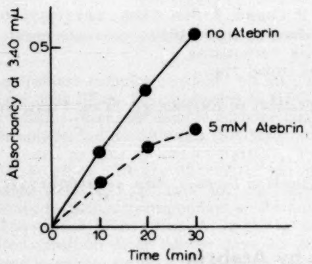

Fig. 1. Inhibition of glucose 6-phosphate dehydrogenase by Atebrin. Each reaction vessel contained: 0.05 *M* Tris–HCl buffer, pH 7.75, 0.02 *M* $MgCl_2$, 0.015 *M* glucose 6-phosphate, 0.0002 *M* TPN, 0.2 ml glucose 6-phosphate dehydrogenase preparation, 0.005 *M* Atebrin (when present), water to a final volume of 2.5 ml. The reaction was stopped by adding NaOH to pH 10 and subsequent boiling. The absorbancy found in a zero-time control was subtracted from the experimental values.

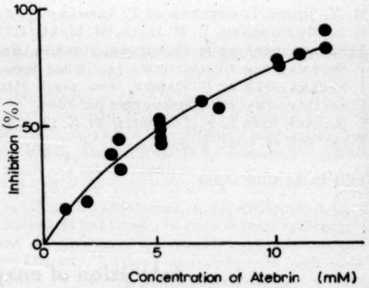

Fig. 2. Inhibition of aliesterase, microsome-bound or soluble, by Atebrin. The activity was measured in Warburg manometers, each vessel containing in the main compartment: 0.1 ml enzyme preparation, varying amounts of Atebrin, 0.025 *M* $NaHCO_3$, water to a final volume of 2.9 ml. After temperature equilibration, the substrate, 0.1 ml 10 % tributyrin, was tipped in from the side arm. Gas phase N_2–CO_2 (95:5).

The inhibition of the microsome-bound ali-esterase by Atebrin or pentachlorophenol was fully reversed by sedimenting the microsomes and washing with 0.5% bovine serum albumin, pH 7.3 or $NaHCO_3$–H_2CO_3 buffer, pH 7.4.

TABLE I

REVERSAL BY FMN OF ATEBRIN INHIBITION OF ALIESTERASE

Atebrin (m*M*)	5	0	5	5
FMN (m*M*)	0	5	5	10
Inhibition (%)	55	0	37	24

Table I shows that inhibition by Atebrin was partly reversed by FMN. During the course of these experiments it was observed that FMN and Atebrin, both of which are yellow, reacted together to form a red-coloured complex. The difference spectrum of the complex is shown in Fig. 3. YAGI[5] has shown that chloropromazine, which is chemically related to Atebrine, as well as other aromatic compounds, forms a complex with flavins.

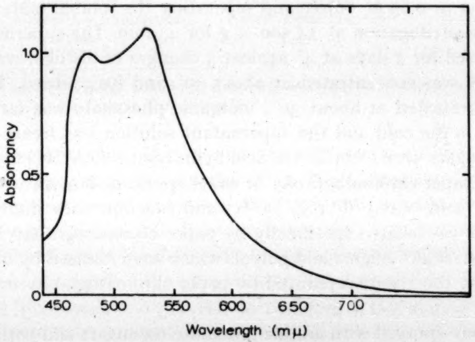

Fig. 3. Difference spectrum: (5 m*M* Atebrin + 5 m*M* FMN) *minus* 5 m*M* Atebrin *minus* 5 m*M* FMN, in Tris–HCl buffer, pH 7.75.

These experiments strongly support HELLERMAN's view[1] that Atebrin is a relatively non-specific enzyme inhibitor, acting by binding with proteins in general.

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[1] L. HELLERMAN, A. LINDSAY AND M. R. BOVARNICK, *J. Biol. Chem.*, 163 (1946) 553.
[2] T. S. WORK AND E. WORK, *The basis of chemotherapy*, Edinburgh and London: Oliver and Boyd, Ltd., 1948, pp. 165, 181.
[3] R. K. MORTON, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. I, New York, Academic Press, Inc., 1955, p. 40.
[4] H. LÖW, *Biochim. Biophys. Acta*, 32 (1959) 1.
[5] K. YAGI, T. OZAWA AND T. NAGATZU, *Nature*, 184 (1959) 982.

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Inhibition by spermine of the action of a bacterial cell-wall lytic enzyme

The cell-wall fraction of a Gram-negative marine bacterium[1] is subject to autodegradation under suitable ionic conditions. This degradation is mediated by an enzyme and is inhibited by the polyamine, spermine, at a concentration of 10^{-3} *M*.

The organism was grown with aeration for 18 h at 30° in sea water containing Difco peptone (1 %, w/v). Cells were harvested, suspended in cold distilled water, and broken by shaking in a Mickle disintegrator for about 18 min with cooling in an ice bath after 9 min. All manipulations in the preparation of the cell walls were performed in an ambient temperature of 4°. The cell-wall fraction was purified by centrifugation in distilled water alternately at 12,200 × *g* for 30 min and 1000 × *g* for 10 min. In general, 4 such cycles were sufficient to yield a cell-wall fraction of acceptable purity. Cell-wall fractions were autolysed at 35° in 0.05 *M* or 0.067 *M* phosphate buffer, pH 8. The extent of the breakdown was followed by measuring changes in absorbancy of the suspensions at 700 mμ. A preliminary qualitative analysis of soluble breakdown products was made after incubating the cell-wall fraction for 35 min in 0.05 *M* buffer and separating the soluble materials from the residual wall by centrifugation at 14,500 × *g* for 45 min. The supernatant solution (lysate) was dialysed for 2 days at 4° against 3 changes of distilled water. The non-dialysable fraction was concentrated at about 30° and freeze-dried. The dialysates were pooled, concentrated at about 30°, inorganic phosphate was largely removed by crystallization in the cold and the supernatant solution was freeze-dried. Amino acids and amino sugars were identified in acid hydrolysates (6 *N* HCl for 16 h at 100°) by 2-dimensional paper chromatography in water–pyridine (80:20, v/v) followed by butan-1-ol–acetic acid–water (6:1:2, v/v/v) and reaction with ninhydrin. α,ε-Diaminopimelic acid was located specifically by paper chromatography in the solvent system of RHULAND *et al.*[2]. Sugars and polyols which were released by acid hydrolysis (2 *N* HCl for 2 h at 100°) were separated by paper chromatography in the butanol–acetic acid solvent system and in propan-1-ol–NH_3 (15 *N*)–water (6:3:1, v/v/v). Such chromatograms were sprayed with aniline phthalate for sugars and with a periodate–Schiff's base reagent[3] for polyols. The heptose present in the cell wall of this organism[1] was detected by the H_2SO_4–cysteine reaction of DISCHE[4].

Fig. 1 shows lysis of the cell wall at pH 8 (properties of the system will be described in greater detail elsewhere) and it will be noted that under these conditions spermine completely inhibited the lytic process. (On the other hand, spermine did not affect the degradation by lysozyme of the cell wall of a species of *Micrococcus*).

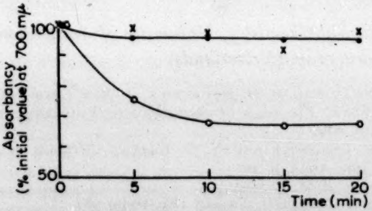

Fig. 1. Autolysis of the cell-wall fraction at 35° in 0.067 *M* phosphate buffer, pH 8. ○, untreated cell walls; ●, heated cell walls (10 min at 100°), ×, cell walls + 10^{-3} *M* spermine.

Table I lists materials released by acid hydrolysis from the cell-wall lysate and the cell-wall residue (or whole cell-wall fraction). It is noteworthy that whereas the "typical" cell-wall constituents, hexosamine and muramic acid, were released, diaminopimelic acid was not. It is also of interest that the dialysable fraction of the lysate consisted of peptides. "Fingerprints"[5] of this fraction separated about 30 peptides, suggesting that the breakdown of the crude cell wall is caused by an enzyme and not merely by spontaneous disintegration under appropriate physico-chemical conditions. The presence in the dialysable fraction of some hydrolysable compound(s) of glycerol probably signifies that the enzyme has degraded lipoprotein. A possible mechanism for the overall breakdown of the cell-wall material might involve enzymic removal of a lipoprotein "cement" followed by spontaneous release of other cell-wall constituents. The inhibition of such a process by spermine provides an interesting extension of MAGER's findings that this substance increases the stability of bacterial cells and spheroplasts against osmotic damage[6-8].

TABLE I

COMPOUNDS DETECTED IN ACID HYDROLYSATES OF THE CELL WALL AND ITS AUTO-DEGRADATION PRODUCTS

Type of compound	*Cell wall (whole and residue)*	*Lysate*	
		Non-dialysable fraction	*Dialysate*
Amino acids	Diaminopimelic acid + normal protein constituents	Normal protein constituents	Alanine Aspartic acid Glutamic acid Glycine Valine or Methionine Leucine or isoleucine
Amino sugars	Hexosamine + muramic acid*	Hexosamine + muramic acid*	—
Sugars	Glucose + heptose	Trace of heptose?	—
Polyols	Glycerol + unidentified polyols	Unidentified polyols	Glycerol

* The proportion of muramic acid to hexosamine was judged by inspection of chromatograms to be greater in the lysate than in the cell walls.

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1 A. D. BROWN, *J. Gen. Microbiol.*, 23 (1960) in the press.
2 L. E. RHULAND, E. WORK, R. F. DENMAN AND D. S. HOARE, *J. Am. Chem. Soc.*, 77 (1955) 4844.
3 J. G. BUCHANAN, C. A. DEKKER AND A. C. LONG, *J. Chem. Soc.*, (1950) 3162.
4 Z. DISCHE, *J. Biol. Chem.*, 204 (1953) 983.
5 V. M. INGRAM, *Biochim. Biophys. Acta.*, 28 (1958) 539.
6 J. MAGER, *Nature*, 176 (1955) 933.
7 J. MAGER, *Biochim. Biophys. Acta*,. 36 (1959) 529.
8 J. MAGER, *Nature*, 183 (1959) 1827.

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Reversible phenomena associated with a helix-coil transition in keratin

If wool-fibre keratin is treated at room temperature in aqueous LiBr of a sufficiently high concentration its X-ray α-pattern is destroyed, but returns if the salt is removed by washing the fibres in water[1]. Furthermore HALY AND GRIFFITH[2] demonstrated that supercontraction of wool fibres in LiBr at 100° takes place in two stages and that the first stage, which could be completed but not exceeded at room temperature, is marked by loss of birefringence. Both length and birefringence are precisely restored if the fibres are washed in water.

We have now found that first-stage supercontraction, birefringence, and the X-ray-diffraction pattern all show an abrupt change over the same small range of LiBr concentration.

All LiBr solutions were prepared by diluting aliquots of a standardised 8.0 M solution. Wool samples for X-ray-diffraction experiments were made up using 50 to 100 Corriedale fibres. A different sample was used for each LiBr solution, and was left in the solution at room temperature for at least 16 h. (No further changes have been observed in times up to many weeks.) After being straightened in a small jig, the fibre bundle and jig were placed in a perspex cell with mica windows (see inset to Fig. 1). The bundle was left wet with LiBr solution, and enough of the same solution was added to the cell to fill it to just below the level of the windows. The object was to maintain the correct relative humidity in the enclosure, so that the concentration of LiBr within the fibres did not alter. After being sealed the cell was placed in the camera of the X-ray-diffraction apparatus (Philips Type PW 1008). An exposure time of 20 min was required with a specimen to film distance of 3 cm and Kodirex film.

Supercontraction was measured using snippets of at least three fibres. These were mounted in the way previously described[2], and left for at least 16 h before measurement.

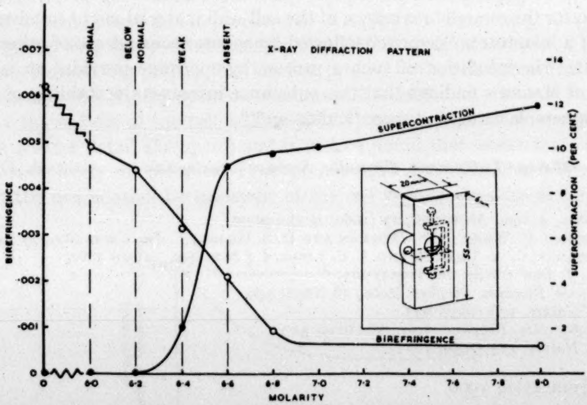

Fig. 1. Curves showing how degree of supercontraction (ordinates at right) and birefringence of wool fibres vary with concentration of aq. LiBr. Only first-stage supercontraction is involved. Qualitative statements about the intensity of the X-ray reflections (5.1 Å and 9.8 Å) are included. A drawing of the cell used for containing the sample during X-irradiation is in the inset.

Birefringence measurements by the method of GORANSON AND ADAMS[3] were made on these same snippets immediately after measurement of degrees of supercontraction. In calculating the birefringence the diameter of the water-saturated fibre was used, *i.e.* no correction was made for swelling due to absorption of LiBr or to longitudinal contraction. The correction is insignificant in the present context.

The results are shown in Fig. 1. Though we have been able to make only qualitative remarks about the loss of the α-pattern, it is clear that all three phenomena show a sharp change over the concentration range 6.2 to 6.6 *M*. There was no evidence from the X-ray photographs that disorientation of crystallites with respect to each other takes place, as arc lengths were the same in all photographs in which the pattern could be seen; this suggests that the structure was disorganized at the molecular level. In addition, extension of a contracted sample in LiBr to a length 50 % greater than the natural length gave no discrete X-ray reflections.

It is important to remember that keratin fibres which are supercontracted, to the end of the first stage in LiBr behave as elastomers[4], *i.e.*, the stress in an extended fibre is entropic and not due to strained bonds. ELÖD AND ZAHN had previously found elastomeric behaviour of keratin under other conditions. These results imply the presence of permanent cross-links separated by many residues, and few or no intra-chain H-bonds.

We thus have evidence that LiBr solution causes disorganization at the molecular level, and that this is accompanied by supercontraction and chain freedom over many residues. A reasonable conclusion would be that inter- and intra-chain hydrogen bonds are ruptured, and that the chains then tend towards the random-coil form. Disorganization at room temperature is not so complete that return to the original configuration is forbidden once the salt is washed out. Such a transition is consistent with the data presented here.

To set against this we have the evidence of HARRINGTON AND SCHELLMAN[6] who worked with solutions of silk fibroin, serum albumin, clupein, and oxidized and native ribonuclease, and deduced that LiBr, while able to destroy the tertiary structure, actually increased the stability of intramolecular H-bonds. Their work was chiefly in the field of optical rotation. STRYER[7] remarked that the increased efficiency of energy transfer in chymotrypsinogen in LiBr, found by SHORE AND PARDEE[8], was consistent with the view that LiBr strengthens peptide H-bonds.

HARRINGTON AND SCHELLMAN concluded that LiBr was a "contra-denaturant", in the sense that it appeared to increase the amount of protein in the α-form. SIKORSKI[9] has, however, reported or quoted cases in which at least partially analogous increases in quantity of α-protein were produced by the action of urea on myosin, and by the action of urea, NaOH, LiBr, formic acid, or cuprammonium hydroxide on denatured keratin. Detection of the α-state was by X-ray diffraction. The denaturation was usually produced by the action of hot water on extended fibres, but in one case it was produced by high-pressure steam which caused supercontraction. (The properties of the native fibre were not restored by the treatment giving additional α-protein.) Of the above reagents at least urea and formic acid are regarded as denaturants.

Thus when results for solid proteins and proteins in solution are considered together some apparent contradictions are found. In an attempt to reconcile the data the following suggestions are made. (a) In solid keratin large numbers of new, small segments of helix might be produced by the LiBr and these would go undetected by the X-ray-diffraction technique whereas a similar phenomenon in solution is seen by optical-rotation methods. The disappearance of the original α-pattern might be explained in terms of small distortions not requiring wholesale breakdown of the secondary structure. It is not clear how supercontraction and elastomeric behaviour would be explained on this basis. (b) In solid protein the reaction due to LiBr may be very different from the reaction in solution. LiBr is absorbed in substantial quantity by the solid and its penetration alone causes a physical disturbance which must involve some forcing apart of molecular chains. We thus have physical and chemical effects to consider and both inter-chain and intra-chain bonds might be weakened or broken. In solution the question of penetration in the same sense does not arise and H-bonds may increase in stability.

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1 E. Hambraeus and R. Steele, *Congres International des Sciences Appliquées à l'Industrie Textile, Ghent 1951*, Paper TO8.
2 A. R. Haly and June Griffith, *Textile Research J.*, 28 (1958) 32.
3 R. W. Goranson and L. H. Adams, *J. Franklin Inst.*, 216 (1933) 475.
4 A. R. Haly and M. Feughelman, *Textile Research J.*, 27 (1957) 919.
5 E. Elöd and H. Zahn, *Melliand Textilbr.*, 30 (1949) 17.
6 W. F. Harrington and J. A. Schellman, *Compt. rend. trav. lab., Carlsberg Sér. chim.*, 30 (1957) 167.
7 L. Stryer, *Biochim. Biophys. Acta*, 35 (1959) 242.
8 V. G. Shore and A. B. Pardee, *Arch. Biochim. Biophys.*, 62 (1956) 355.
9 J. Sikorski, *Nature*, 170 (1952) 275.

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The application of the Sakaguchi reaction to the determination of DNP-arginine

During the study of an N-terminal amino acid residue of a protein by the dinitrophenylation procedure[1] it is necessary, when the end-group is arginine, to be able to determine accurately the amount of DNP-arginine present in the mixture of amino acids resulting from the acid hydrolysis of the dinitrophenylated protein. A method whereby this may be achieved has been outlined by Wissmann and Nitschmann[2]. In this, the DNP-arginine is first separated from the accompanying free amino acids by chromatography on talc columns, and subsequently subjected to a modification[3] of the Sakaguchi reaction, the colour produced being compared with that from similarly treated standards. The validity of the determination is therefore dependent on the assumption that DNP-arginine behaves similarly to arginine when treated with alkaline hypobromite.

When attempts were made to determine the DNP-arginine released by hydrolysis of dinitrophenylated α-casein using this method, very variable results were obtained. It was found that in order to obtain reproducible results with DNP-arginine, the rate

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Abbreviation: DNP-, 2,4-dinitrophenyl-.

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of addition of the reactants, and the time allowed for colour development required more strict control than was apparently necessary for the accurate determination of arginine itself. This stricter control has been incorporated in MACPHERSON's modification[3] of the Sakaguchi reaction, as follows.

Portions of a solution of DNP-arginine in 0.1 *N* HCl, usually about 3 ml and containing 50–500 μg DNP-arginine, are neutralised by addition of 10 % KOH, and the addition continued until an excess of 1 ml has been added. This is followed by 2 ml of 0.1 % α-naphthol in 50 % ethanol, and 1 ml 40 % aq. urea. Water is then added until the final volume of each mixture is 14 ml. To each, 1 ml of a solution containing 2 g bromine in 100 ml 5 % KOH is now added in a dropwise manner and with shaking, over exactly 25 sec. After a further 2 min, 1 ml of 40 % urea is added followed by a second addition of 1 ml of the hypobromite solution, again over 25 sec. The reaction mixture is allowed to stand at room temperature for 10 min and is then diluted to 25 ml with water. After a further 10 min the light absorption of the solution at 525 mμ is measured against a blank determination containing no DNP-arginine. The colour produced increased linearly with weight of DNP-arginine up to at least 1500 μg, and was sufficiently intense for reproducible results to be obtained from as little as 70 μg.

However, DNP-amino acids especially in solution are subject to photolytic decomposition[4–7], and reliable results were obtained with the above method only when the DNP-arginine was of recent preparation, and the solutions employed were shielded from light. In an attempt to eliminate the necessity for these precautions, and to provide a stable and more easily purified standard material, the determination was performed with L-arginine. It was found that while the results were again reproducible, the amount of colour produced by pure DNP-arginine was in excess of that produced by a molecular equivalent of L-arginine by between 40 and 100 % depending upon the exact conditions used. Furthermore, the wavelength of maximum light absorption found for the DNP-arginine colour was approximately 525 mμ whereas that of the L-arginine colour was 505 mμ, as shown in Fig. 1. It might be supposed that this enhanced value for DNP-arginine is due directly to the presence of the dinitrophenyl chromophore whose wavelength of maximum light absorption is approximately 355 mμ. That this is not so can be seen from Fig. 1 where DNP-arginine in alkaline solution is shown to exhibit no selective light absorption in the region of 525 mμ. It is also apparent that although DNP-arginine obtained by hydrolysis of a dinitrophenylated protein is not separated by chromatography on talc from the accompanying ε-DNP-lysine, 2,4-dinitrophenol, and 2,4-dinitroaniline, the DNP groups of these substances having no absorption at 525 mμ cause no interference in the determination.

It was concluded that the difference in colour production between arginine and DNP-arginine, which makes the use of L-arginine as a standard undesirable, is due to differences in reactivity of the guanido groups in the two molecules brought about by the presence in one of them of the DNP group, at a point remote from the site of the primary reaction. To check this, two further substituted guanidines, methyl guanidine ($CH_3NHC[=NH]NH_2$), and guanidoacetic acid ($HOOCCH_2NHC[=NH]NH_2$), were treated with hypobromite under the same conditions used for DNP-arginine and L-arginine. It was found that while the density of the colour produced by L-arginine was almost identical with that from a molecular equivalent of guanidoacetic acid it was again less than that produced by DNP-arginine, and very much less than that from methyl guanidine. This is illustrated in Fig. 2. These findings can be explained by supposing that there is a weak interaction between the acidic and basic extremities of the arginine and guanidoacetic acid molecules which prevents full colour development in the Sakaguchi reaction. No such interaction is possible with methylguanidine, while in DNP-arginine the presence of a substituent as large as the dinitrophenyl group in close proximity to the carboxyl group might be expected to prevent or at least hinder such an interaction. This being so, the guanido groups of both substances would be free to react more easily with alkaline hypobromite than the corresponding grouping in either L-arginine or guanidoacetic acid.

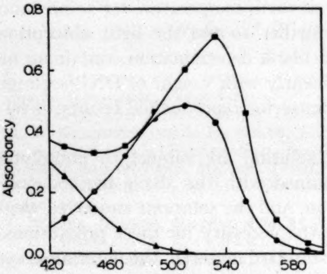

Fig. 1. ▲, absorption spectra of DNP-arginine in alkaline solution; ●, products resulting from Sakaguchi reaction on arginine and ■, DNP-arginine. In each the concentration of arginine derivative was 1.10 μmoles/25 ml.

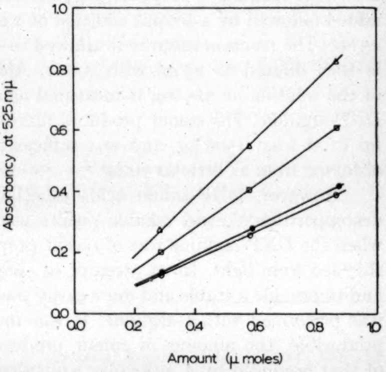

Fig. 2. Colour produced by Sakaguchi reaction from; ○, arginine; ●, guanidoacetic acid; □, DNP-arginine and △, methyl guanidine.

In summary, application of the Sakaguchi reaction to the determination of DNP-arginine has yielded results of sufficient accuracy to be valuable in the study of protein end-groups, only when the precautions and modifications outlined above have been observed, and in particular when the standards employed were prepared from DNP-arginine of the highest purity, *i.e.* having m.p. 258–259° and $\varepsilon_{\max} = 17{,}600$ at 355 mμ in 1% NaOH.

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[1] F. Sanger, *Biochem. J.*, 39 (1945) 507.
[2] H. Wissmann and Hs. Nitschmann, *Helv. Chim. Acta*, 40 (1957) 356.
[3] H. T. MacPherson, *Biochem. J.*, 36 (1942) 59.
[4] B. Pollara and R. W. Von Korff, *Biochim. Biophys. Acta*, 39 (1960) 364.
[5] F. Sanger, *Biochem. J.*, 45 (1949) 563.
[6] S. Blackburn, *Biochem. J.*, 45 (1949) 579.
[7] G. L. Mills, *Biochem. J.*, 50 (1952) 707.

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Preliminary Notes

Affinity of N-acetylneuraminic acid for influenza virus neuraminidase

It is well known[1,2] that neuraminidases from different sources split off NANA from several substrates. As observed in our laboratory[3] and confirmed by FAILLARD[4] and ROMANOWSKA[5], influenza-virus neuraminidase from several strains can be inhibited by NANA. Using our techniques[3], it can be shown that several sugars (including lactose) occurring in the substrates of this enzyme are without effect on the enzymic activity.

To obtain more quantitative data about the relationships involved, an accurate chemical determination of neuraminidase activity was necessary. Using WARREN's thiobarbituric acid assay[6] for free NANA such a method was worked out by JACOBS AND WALOP[7]. This method made it possible to study the interaction of neuraminidase and its substrates. However, in studies on the inhibition of neuraminidase by NANA, where amounts of NANA are added in excess of that released enzymically, the above-mentioned method does not permit a reliable interpretation. For this reason a method was developed in which instead of the NANA released by the enzyme, the NANA remaining in the substrate is determined. As HEIMER[8] observed, it is possible to reduce NANA by $NaBH_4$ to a compound, which—in contrast to NANA—does not act as a chromogen in the colour reaction with Ehrlich's *p*-dimethylaminobenzaldehyde reagent. Since ketosidically-bound NANA is not reduced by $NaBH_4$, a differentiation can be made in this way between free NANA—enzymically released or added—and ketosidically-bound NANA remaining in the substrate.

The substrate used in this study was N-acetylneuraminyl-lactose, prepared from cow colostrum by a procedure rather similar to the isolation of N-acetyl-O-acetylneuraminyl-lactose[9]. The determinations of neuraminidase activity were carried out in the following way: mixtures of equal volumes of 0.025 *M* phosphate buffer, substrate solution (N-acetylneuraminyl-lactose in concentrations varying from 100 to 400 μg/ml in phosphate buffer) and neuraminidase solution are incubated at 37°. The pH of all solutions is 6.8. In inhibition studies the volume of phosphate buffer is replaced by an appropriate buffered solution containing the inhibitor. At various intervals, starting at zero time, 1.50-ml aliquots of the incubation mixture are transferred to glass-stoppered tubes containing 0.20 ml of a solution of 2 mg ethylenediaminetetraacetate (to stop the reaction) and 16 mg $NaBH_4$ in 0.1 *N* NaOH. The reduction of the free NANA is allowed to proceed for 30 min at room temperature, after which time 0.20 ml *N* HCl is added to decompose the excess $NaBH_4$. The remaining NANA is then determined with Ehrlich's reagent, using the procedure of WERNER AND ODIN[10], slightly modified.

This method was used to study the interaction of crystalline NANA and purified influenza-virus particles of the strain A_2 1 (1957 Singapore). Fig. 1 shows the results of a typical experiment, plotted according to DIXON[11] to determine both the type of inhibition and the inhibitor constant. From this figure, it can be inferred that:

Abbreviation: NANA, N-acetylneuraminic acid.

(a) the system influenza-virus and N-acetyl-neuraminyl-lactose follows Michaelis–Menten kinetics; (b) the inhibition by NANA is of the competitive type; (c) the inhibitor constant (K_i), representing the reciprocal of the affinity of NANA to influenza-virus neuraminidase, can be calculated. The mean value of K_i was determined as $5 \cdot 10^{-3}$ M.

With our method, based on the determination of NANA release[7], we determined the K_m of the system influenza-virus neuraminidase and N-acetylneuraminyl-lactose. Using the LINEWEAVER–BURK plot[11], as shown in Fig. 2, K_m can be estimated to be $6 \cdot 10^{-4}$ M. If we assume that K_m is a close approximation to the dissociation constant of the enzyme–substrate complex, comparison of this K_m for N-acetylneuraminyl-lactose with the K_i for NANA would indicate that the affinity of the NANA molecule for the enzyme surface is increased 8-fold by the ketosidic linkage of the NANA moiety to lactose.

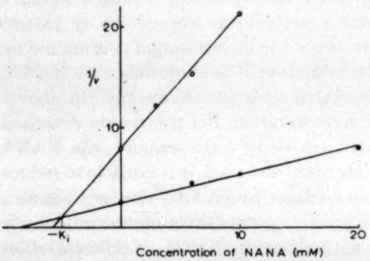

Fig. 1. Dixon plot for type of inhibition and determination of inhibitor constant, using influenza virus A_2 1 (1957 Singapore) as neuraminidase. N-acetylneuraminyl-lactose concentrations, 125 μg/ml (○—○) and 400 μg/ml (●—●). The units on the $1/v$ axis are arbitrary.

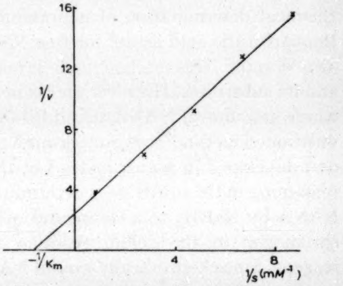

Fig. 2. Lineweaver–Burk plot for determination of Michaelis–Menten constant, using influenza virus A_2 1 (1957 Singapore) as neuraminidase. The units on the $1/s$ axis are the reciprocals of the mM concentrations of N-acetylneuraminyl-lactose. The units on the $1/v$ axis are arbitrary.

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[1] A. GOTTSCHALK, *Advances in Enzymol.*, 20 (1958) 135.
[2] F. ZILLIKEN AND M. W. WHITEHOUSE, *Advances in Carbohydrate Chem.*, 13 (1958) 237.
[3] J. N. WALOP, *Abstr. Communs. 4th Intern. Congr. Biochem., Vienna* 1958.
[4] H. FAILLARD, *Z. physiol. Chem.*, 317 (1959) 257.
[5] E. ROMANOWSKA, *Naturwiss.*, 47 (1960) 66.
[6] L. WARREN, *J. Biol. Chem.*, 234 (1959) 1971.
[7] J. JACOBS AND J. N. WALOP, *Nature*, (1960), in the press.
[8] R. HEIMER, *Thesis*, Columbia University, University Microfilms, Inc., Ann Arbor 1958.
[9] R. KUHN AND R. BROSSMER, *Chem. Ber.*, 89 (1956) 2013.
[10] I. WERNER AND L. ODIN, *Acta Soc. Med. Upsaliensis*, 57 (1952) 230.
[11] M. DIXON, *Biochem. J.*, 55 (1953) 170.
[12] H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.

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The scope of amino donor specificity of glutamine transaminase and asparagine transaminase

Transamination of the α-amino group of glutamine (and some closely related analogs) and of asparagine is catalysed by two enzymes, glutamine transaminase and asparagine transaminase, discovered in rat liver by MEISTER *et al.*[1,2] and currently known to occur in many biological systems, including plants and bacteria. Both enzymes transfer the $-NH_2$ group to a large number of various α-keto acids. In contrast to their broad acceptor specificity, these transaminases are generally believed to have narrow specificity with respect to the $-NH_2$ donor[3].

The peculiar substrate specificity assumed for these so-called amino acid amide α-keto acid transaminases does not agree with some evidence indicating reversibility of their action[4]. It is in conflict with the well-established, generally accepted "shuttle" mechanism of enzymic transamination, postulating two alternating binary half-reactions in which the transaminase acts as intermediary $-NH_2$ acceptor[5-7]. This mechanism carries the implication that a transaminase should catalyse reversible transamination reactions between the α-amino analogs of all α-keto acids that can accept $-NH_2$ groups from the pyridoxamine form of the enzyme, and any of these keto acids[7].

If the above mechanism is valid, then glutamine transaminase and asparagine transaminase should act, in accordance with the scope of their acceptor specificity[1-3], as fairly non-specific α-amino-α-ketomonocarboxylate transaminases.

This is probably in fact the case, as indicated by the following preliminary data obtained in experiments with extensively purified amino acid amide transaminases from rat liver.

The purification procedures developed by the authors[8], involving heat treatment, $(NH_4)_2SO_4$ fractionation, zone electrophoresis and adsorption on DEAE-cellulose, eliminate glutaminase, ketoamidase, most of the contaminating transaminases, and separate the glutamine and asparagine transaminases from each other. As compared with crude liver extract, the activity of the partially purified glutamine enzyme is raised 50- to 80-fold and that of the asparagine enzyme, 30- to 40-fold. The main, active component of each preparation (50–70% of the total protein) is electrophoretically homogeneous; the maximal rates of transamination are fairly low as compared, for example, with alanine–glutamate transaminase.

With these enzyme preparations, the "adequate" amides and different α-amino acids were tested as $-NH_2$ donors for transamination with pyruvate, phenylpyruvate and α-ketoisocaproate.

The experimental results presented in Table I clearly show that both amide transaminases effectively catalyse $-NH_2$ transfer from a number of α-aminomonocarboxylic acids (at increased concentration levels) to pyruvate, phenylpyruvate and α-ketoisocaproate. With enzymes of animal origin, transaminations to the latter two keto acids from donors other than alanine, glutamate, glutamine or asparagine have not been demonstrated before.

The reaction rates were low with 14 mM α-aminomonocarboxylic acids, which indicates that their K_S values for the two transaminases studied are considerably higher than those of the "adequate" amino acid amides.

Abbreviations: DEAE-, diethylaminoethyl-; EDTA, ethylenediaminetetraacetate.

TABLE I

TRANSAMINATIONS CATALYSED BY "AMINO ACID AMIDE" TRANSAMINASES

Amino donors	*Glutamine transaminase* *Amino acceptors*			*Asparagine transaminase* *Amino acceptors*		
	pyruvate	*phenylpyruvate*	*α-ketoisocaproate*	*pyruvate*	*phenylpyruvate*	*α-ketoisocaproate*
	Amino group transfer (μmoles/mg enzyme protein)					
L-glutamine, 14 m*M*	—	5.4	—	—	0.15	—
L-glutamine, 67 m*M*	—	—	—	—	0.60	—
L-asparagine, 14 m*M*	—	0.18	—	1.90	1.70	—
L-asparagine, 67 m*M*	—	0.54	—	—	—	—
L-alanine, 67 m*M*	—	1.20	—	—	1.29	—
DL-aminobutyrate, 67 m*M*	3.75***	1.50	1.08	0.47	0.40	0.34
L-leucine, 67 m*M*	1.62	*	—	0.82	*	—
DL-norleucine, 67 m*M*	2.42	*	*	0.82	*	*
L-methionine, 67 m*M*	2.70	2.92	3.09	1.10	1.50	1.15
L-histidine, 67 m*M***	2.52	2.14	2.43	0.84	1.32	1.34
L-phenylalanine, 67 m*M*	2.22	—	*	0.73	—	*
L-glutamate, 67 m*M*	10.10***	0	0.88	4.10***	0	0.07

The enzyme preparations (0.3–0.5 mg glutamine transaminase, or 0.8–1.0 mg asparagine transaminase) were incubated for 2 h at 37° in 1 ml 0.05 *M* veronal buffer (pH 8.4) with the substrates indicated (28 m*M* pyruvate, 14 m*M* phenylpyruvate, 28 m*M* α-ketoisocaproate). Deproteinized filtrates of the incubated samples and blanks with omitted $-NH_2$ donor or acceptor were subjected to descending paper chromatography with appropriate solvent mixtures, followed by quantitative photometric estimation of the amino acids formed by transamination (alanine, phenylalanine or leucine) as the ninhydrin–copper complexes[8]. The $-NH_2$ transfer values presented are corrected by subtraction of the higher of the two incomplete blanks mentioned above.

* Chromatographic separation of amino acids inadequate; values omitted.

** Samples supplemented with 10^{-4} *M* EDTA to inhibit histidine deaminase.[2]

*** $-NH_2$ group transfer high, owing to the presence of contaminating L-alanine–glutamate transaminase.

It is worthy of note that the amide transaminases also manifest slow cross-reactions with increased concentrations of the "inadequate" amino acid amide (*cf.* ref. 9).

When asparagine transaminase was incubated with phenylpyruvate and non-saturating concentrations of (a) asparagine, (b) L-alanine, and (c) a mixture of both $-NH_2$ donors, phenylalanine formation was competitive rather than additive, *viz.*: a, 1.21; b, 1.50; and c, 1.66 μmoles phenylalanine. These data prove that the transamination of alanine was due to the asparagine transaminase and not to contamination of this enzyme preparation with phenylalanine–pyruvate transaminase.

It thus appears that the two liver enzymes discovered by MEISTER *et al.* in studies on the coupled transamination of glutamine and asparagine and hitherto considered as specific amino acid amide transaminases probably are, in fact, group-specific general monocarboxylate transaminases. The classification of these enzymes as glutamate and asparagine transaminases may be justified insofar as their affinity to the corresponding amino acid amides is, apparently, markedly higher than to other amino-monocarboxylate donors. However, the present evidence for a broad scope of donor as well as acceptor specificity of these transaminases calls for reinvestigation of a number of claims concerning the independent existence of enzymes specifically catalysing miscellaneous transamination reactions.

Further studies are under way on the substrate affinities and specifities of glutamine and asparagine transaminases.

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[1] A. MEISTER AND S. V. TICE, *J. Biol. Chem.*, 187 (1950) 173.
[2] A. MEISTER, H. A. SOBER, S. V. TICE AND P. E. FRASER, *J. Biol. Chem.*, 197 (1952) 319.
[3] A. MEISTER, *Advances in Enzymol.*, 16 (1955) 185; *Biochemistry of the Amino Acids*, Academic Press, New York, 1957, p. 179.
[4] A. MEISTER AND P. E. FRASER, *J. Biol. Chem.*, 210 (1954) 37.
[5] F. SCHLENK AND A. FISHER, *Arch. Biochem.*, 12 (1947) 62.
[6] A. E. BRAUNSTEIN, in *The Enzymes*. ed. by P. BOYER, H. LARDY AND K. MYRBÄCK, Vol. 2, Academic Press, Inc., New York, 1960, p. 113.
[7] E. E. SNELL AND W. T. JENKINS, *J. Cellular Comp. Physiol.*, 54 Suppl. 1 (1959) 161.
[8] A. E. BRAUNSTEIN AND HSU TING SENG, *Biokhimiya*, 25 (1960), 758.
[9] HSU TING SENG AND M. LERMAN, (1960) in preparation.

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Terminal incorporation of [^{14}C]AMP into s-RNA by bacterial enzymes

AMP, derived from ATP, is incorporated into terminal positions of soluble ribonucleic acid (s-RNA) by enzymes derived from rat liver. This attachment of AMP has been shown to occur predominantly in positions adjacent to cytidylic acid of s-RNA, thus establishing the terminal ribonucleotide sequence and the specificity of the reaction[1]. The enzyme fractions responsible for the incorporation of terminal ribonucleotides into s-RNA have been obtained primarily from extracts of rat liver[1–4].

Since an analogous type of s-RNA has been found to exist in *Escherichia coli*[5], the presence of enzymes associated with its synthesis has been sought in extracts of this microorganism. The availability of enzyme systems from two markedly different sources (animal and bacterial) responsible for the same reaction would permit a detailed comparative study and lead to a better understanding of these reactions. In the present communication the existence is demonstrated in *E. coli* of an enzyme system responsible for the terminal incorporation of [^{14}C]AMP from [^{14}C]ATP into s-RNA, in positions adjacent to cytidylic acid; this enzyme system has been partially purified.

Extracts of *E. coli*[6] were centrifuged for 1 h at 100,000 × *g* and the pH of the supernatant fraction was adjusted to 3.8. The resulting isoelectric precipitate (Table I, Fraction 1) retained more than 90 % of the ability of the crude extract to incorporate [^{14}C]AMP into RNA when [^{14}C]ATP was the precursor (results not shown). As shown in Table I (Fraction 1), after alkaline hydrolysis of the isolated radioactive RNA, 60 % of the incorporated [^{14}C]AMP was liberated as adenosine and 40 % as AMP. Further purification resulted in the isolation of a fraction (Table I, Fraction 2)

Abbreviations: AMP, ADP, ATP, adenosine mono-, di- and triphosphate; s-RNA, soluble ribonucleic acid.

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN ALKALINE HYDROLYSATES OF s-RNA

The incubation mixture consisted of 30 mμmoles of [^{14}C]ATP or [γ-^{32}P]ATP, of specific activity 0.88 μC/μmole and 5.0 μC/μmole, respectively, 100 μmoles tris(hydroxymethyl)aminomethane-HCl buffer, pH 8.0, 20 μmoles $MgCl_2$, 5 μmoles $MnCl_2$, 18 μmoles creatine phosphate, 0.2 mg creatine kinase[10], and 0.8 mg of *E. coli* enzyme; final volume, 1.3 ml. After incubation at 37° for 20 min, the samples were chilled in ice, and 8 ml of cold 0.4 N $HClO_4$ were added. The precipitates were washed and hydrolyzed with 0.3 N KOH overnight at 37°. The resultant acid-soluble fraction was fractionated by column chromatography[12].

Substrate	*Enzyme*	*Radioactivity in:*				
		Adenosine	*Cytidylic*	*Uridylic*	*Adenylic*	*Guanylic*
		as % of total				
[^{14}C]ATP	Fraction 1	60	—	—	40	—
[^{14}C]ATP	Fraction 2a	90–95	—	—	5–10	—
[γ-^{32}P]ATP	Fraction 2a	—	87.5	3.5	6.5	2.5
[γ-^{32}P]ATP	Fraction 2b	—	85.5	3.5	9.5	1.5

(a) 23.0 A_{260} units of *E. coli* s-RNA were added.
(b) 6.0 A_{260} units of s-RNA-β + -γ were added.

which incorporated [^{14}C]AMP into RNA in significant amounts only if s-RNA was added. In this case, the [^{14}C]AMP was incorporated mainly in terminal positions, as evidenced by the fact that, after alkaline hydrolysis of the radioactive s-RNA, 90–95 % of the incorporated radioactivity was recovered as adenosine. If a similar experiment was performed using [γ-^{32}P]ATP as the substrate, the ^{32}P in the alkaline hydrolysate of the isolated s-RNA was primarily associated with 2′(3′)-cytidylic acid. This occurred whether the s-RNA added was derived from *E. coli* or from rat liver. The s-RNA used in the latter case (s-RNA-β + -γ)[4,7] is known to contain only about 15 % terminal cytidylic acid groups; the remaining 85 % of the polynucleotide is composed primarily of adenylic acid and guanylic acid, with some uridylic acid being present. Thus, the bacterial enzyme system shows a distinct preference for attaching adenylic acid to cytidylic acid groups in terminal positions.

In order to ascertain the nature of the precursor for this reaction, the enzyme system was incubated with [^{14}C]ATP or [^{14}C]ADP, in the presence and absence of a phosphorylating system. The results presented in Table II indicate that optimal incorporation occurred when the phosphorylating system was present. The slight incorporation which took place in the presence of [^{14}C]ADP alone can be explained by myokinase activity, which is known to contaminate the enzyme preparation. Analysis of the acid-soluble fractions under these conditions showed appreciable degradation of the added [^{14}C]ATP, incubated in the absence of creatine phosphate and creatine kinase, as well as of the added [^{14}C]ADP; in the latter case, some [^{14}C]ATP was also formed. In the presence of the phosphorylating system, [^{14}C]ATP was the major constituent of the acid-soluble fractions. Accordingly, these results provide strong evidence that [^{14}C]ATP is the precursor in the reaction under study.

These experiments indicate that enzymes associated with the synthesis of s-RNA, which are essentially similar to those previously found in animal tissues[1–3] and probably similar to those recently reported[8,9], have been purified from bacterial

TABLE II

THE ABILITY OF [^{14}C]ADP AND OF [^{14}C]ATP TO SERVE AS PRECURSORS

The basic incubation mixture was the same as that described in Table I, except for the omission of creatine kinase and creatine phosphate from those samples marked (—). All samples contained 23.0 A_{260} units *E. coli* s-RNA. When [^{14}C]ADP was used, 30 μmoles were added (specific activity, 0.65 μC/μmole). After incubation at 37° for 20 min, 8 ml cold 0.4 N $HClO_4$ were added; the precipitates were washed repeatedly, plated and counted[11].

Substrate	*Creatine kinase + Creatine phosphate*	*[^{14}C]AMP incorporated mμmole*
[^{14}C]ATP	+	0.25
[^{14}C]ATP	—	0.18
[^{14}C]ADP	+	0.23
[^{14}C]ADP	—	0.09

extracts. Detailed comparative studies of the enzymes from these two sources will be presented at a later date.

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[1] E. S. CANELLAKIS, *Biochim. Biophys. Acta*, 25 (1957) 217.
[2] L. I. HECHT, P. C. ZAMECNIK, M. L. STEPHENSON AND J. F. SCOTT, *J. Biol. Chem.*, 233 (1958) 954.
[3] E. HERBERT, *J. Biol. Chem.*, 231 (1958) 975.
[4] E. S. CANELLAKIS AND E. HERBERT, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 170.
[5] S. LACKS AND F. GROS, *J. Mol. Biol.*, 1 (1959) 301.
[6] E. S. CANELLAKIS, M. E. GOTTESMAN AND H. O. KAMMEN, *Biochim. Biophys. Acta*, 39 (1960) 82.
[7] E. HERBERT AND E. S. CANELLAKIS, *Biochim. Biophys. Acta*, in the press.
[8] T. PREISS AND P. BERG, *Federation Proc.*, 19 (1960) 317.
[9] M. ALEXANDER, A. BRESLER, J. FURTH AND J. HURWITZ, *Federation Proc.*, 19 (1960) 318.
[10] S. A. KUBY, L. NODA AND H. LARDY, *J. Biol. Chem.*, 209 (1954) 191.
[11] E. S. CANELLAKIS AND E. HERBERT, *Biochim. Biophys. Acta*, in the press.
[12] E. S. CANELLAKIS AND R. MANTSAVINOS, *Biochim. Biophys. Acta*, 27 (1958) 643.

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Digestion of ribonuclease A with chymotrypsin and trypsin at high temperatures

We should like to describe some experiments which offer the possibility of locating the non-helical regions in native ribonuclease and also the portions of the polypeptide chain involved in the reversible, thermal transition[1]. These experiments were based on the hypothesis that the unfolded parts of a polypeptide chain are more susceptible than the helical portions to attack by proteolytic enzymes[2]. If the amino acid sequence is known, the positions of the enzymic cleavages may be determined. In contrast to subtilisin, which hydrolyzes a specific alanyl–seryl bond in ribonuclease A[3], both chymotrypsin and trypsin fail to hydrolyze the native protein[4]. Recent work in this laboratory[5] on the pH-dependence of the reversible, thermal transition in ribonuclease indicates that the native molecule is only partially unfolded in the transition. The portions of the polypeptide chain involved in this limited configurational change might therefore be located if the unfolding produced weak points in the molecule, *i.e.* bonds which are susceptible to proteolytic attack only under conditions where the protein has been denatured. Previously, guanidine hydrochloride has been used to effect such a structural change permitting enzymic hydrolysis[4]; however, the complications introduced by the use of guanidine can be avoided with a knowledge of the behavior of the thermal transition[5].

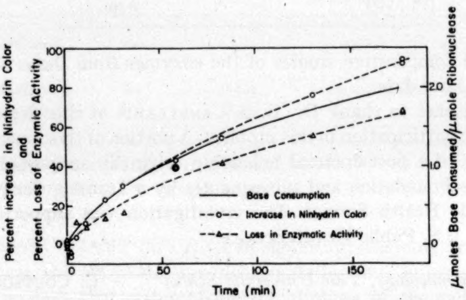

Fig. 1. The hydrolysis of ribonuclease A by chymotrypsin at pH 6.5 at 50°.

In order to locate unfolded regions of ribonuclease A, the protein was digested with chymotrypsin and trypsin at temperatures sufficiently high (40°–50°) to initiate unfolding of the molecule. Fig. 1 shows the progress of a typical hydrolysis by chymotrypsin at pH 6.5 and 50°. The reaction mixture contained 9.7 mg/ml chromatographically purified ribonuclease A, 0.011 mg/ml chymotrypsin, and 0.01 M $CaCl_2$. A control solution of ribonuclease A which was heated at 60° for 100 min in the absence of proteolytic enzymes showed less than a 5 % change in ninhydrin color and enzymic activity. Analogous results were obtained when trypsin was substituted for chymotrypsin. In the experiment above with chymotrypsin the rate of digestion was 250 times that obtained at pH 8.0 at 25°. In another experiment, increasing the temperature from 40.3° to 43.6° resulted in a greater than 5-fold increase in the rate of digestion by chymotrypsin. This large temperature effect indicates that a configurational change in the ribonuclease molecule is most probably responsible for the increased susceptibility to proteolytic attack.

The results of the chromatographic analysis shown in Fig. 2 demonstrate that intermediates accumulate when ribonuclease A is digested with chymotrypsin at 50°. The fraction (VI) containing enzymic activity was eluted at a volume identical with that for native ribonuclease A, as determined in control experiments. The absence of ribonuclease activity in other fractions indicates that the cleavages introduced by chymotrypsin destroy the configuration required for the biological activity of this protein. Preliminary characterization of the various components by gel filtration using Sephadex G-50 indicates that fractions II, V, VI, and VII (Fig. 2) contain material of appreciable molecular size.

Buffers containing 0.1 to 0.5 *M* sodium phosphate or citrate reduced the rate of digestion with chymotrypsin by more than 10-fold; in contrast, 0.5 *M* KCl did not greatly alter the rate. These observations are consistent with the specific effect of phosphate and other polyvalent anions in stabilizing ribonuclease A against urea denaturation[6]. The distributions of components in the chromatographic patterns of the digests made in the presence of phosphate and citrate were significantly different from the patterns of digests made in the absence of these ions.

Work is now in progress on the characterization of the various intermediates formed by digestion of partially unfolded ribonuclease with chymotrypsin and trypsin, respectively. Particular emphasis will be placed on determining the sites of enzymic cleavage, and the effect of these cleavages on the physical and chemical properties of ribonuclease A. It is hoped that the positions of splits in the polypeptide chain, which occur in the early stages of the digestion, will indicate what portions of the sequence are involved in the thermal transition.

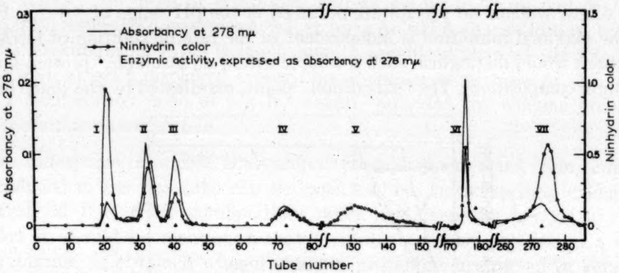

Fig. 2. The chromatographic analysis of ribonuclease A digested with chymotrypsin at pH 6.5 at 50°. The analysis was performed on a 30 × 1-cm column of IRC-50, XE-64 resin; elution was accomplished with 0.15 *M* sodium phosphate, 0.001 *M* ethylenediaminetetraacetate, pH 6.00 until tube 143, then with 1.0 *M* sodium phosphate, 0.001 *M* ethylenediaminetetraacetate buffer, pH 6.47; 0.5-ml fractions were collected and were diluted with 3.0 ml water before analysis.

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[1] W. F. HARRINGTON AND J. A. SCHELLMAN, *Compt. rend. trav. lab. Carlsberg, Ser. Chim.*, 30 (1956) 21.
[2] E. MIHALYI AND W. F. HARRINGTON, *Biochim. Biophys. Acta*, 36 (1959) 447.
[3] F. M. RICHARDS AND P. J. VITHAYATHIL, *J. Biol. Chem.*, 234 (1959) 1459.
[4] D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *J. Biol. Chem.*, 235 (1960) 648.
[5] J. HERMANS, JR., AND H. A. SCHERAGA, *J. Am. Chem. Soc.*, to be submitted.
[6] M. SELA, C. B. ANFINSEN AND W. F. HARRINGTON, *Biochim. Biophys. Acta*, 26 (1957) 502.

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Soluble stoichiometric complexes of DNA with chymotrypsin and trypsin

Chymotrypsin

The hydrolytic activity of crystalline chymotrypsin in 10 ml 0.01 *M* methylhippurate was measured with a pH-stat at pH 7.5 and 30° in the absence of buffer. As shown in Fig. 1, addition of DNA (a "highly polymerized" preparation from calf thymus, Worthington) to the enzyme solution resulted in an inhibition proportional to the DNA concentration and inversely proportional to the ChT concentration up to the maximum of about 80 % inhibition. Such a relationship indicates irreversible binding and suggests the formation of a definite (stoichiometric) complex. Although with higher ChT concentrations an insoluble complex is formed at pH < 7, in the present dilute systems no precipitate occurred in the pH range of 7.5–2.5. Furthermore, the maximal inhibition is independent of the rate of addition of DNA. These observations would distinguish such a complex from an insoluble "protein-nucleate" of indefinite composition[1]. The "saturation" point, manifested by the sharp breaks in the curves of Fig. 1, indicates a protein: DNA weight ratio of about 20:1, corresponding to approximately 4 nucleotide units per ChT molecule. In contrast to DNA, CMC (sodium salt, Hercules) which is also a potent inhibitor, combines in a dissociable ("Michaelis") manner shown by the dotted curve in Fig. 1. Thymus RNA (sodium ribonucleate, Nutritional Biochemicals Inc.) produced results intermediate between the curves of DNA and CMC. Yeast RNA (Pabst Laboratories) gave a curve resembling that of CMC. It must be noted, however, that the properties of the nucleic acids depend on the procedure of preparation[2,3]; *i.e.*, the possibility cannot be excluded that in the native state RNA combines in a manner similar to DNA.

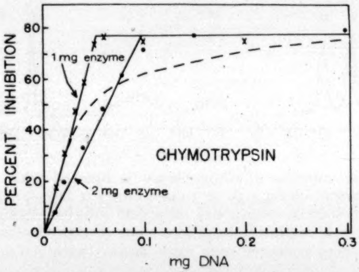

Fig. 1. Inhibition of the hydrolysis of methylhippurate (0.01 *M*) by ChT in concentrations of 1 and 2 mg per 10 ml in the presence of varying amounts of DNA at pH 7.5. The dotted line represents a dissociation curve such as that given by CMC.

Heating neutral solutions of DNA or RNA at 95–100° had little or no influence on the curves, but heating at alkaline pH decreased the inhibition, especially in the case of RNA. Pretreatment of yeast RNA with ribonuclease greatly decreased its inhibitory power. Addition of RNA-ase to the RNA-inhibited ChT was less effective, indicating that combination with ChT protects RNA against attack by RNA-ase. In view of the results of heat treatment and in view of the contention that RNA does

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Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; ChT, chymotrypsin; CMC, carboxymethylcellulose.

not have the typical two-stranded structure of DNA[2–4], it is unlikely that irreversible binding is connected exclusively with such a structure. The evidence rather indicates that it is merely connected with a high degree of polymerization and regularity of structure. These properties seem to be exhibited especially by the better preparations of DNA, in contrast to the available RNA preparations[2,3].

The inhibition by the polymers is prevented by KCl in concentrations of the order of 0.1 *M*. Preliminary results with lower salt concentrations indicate that, at least in the case of DNA, this could partly be caused by an increase of the enzymic activity of the complex instead of a dissociation into free enzyme and nucleic acid.

Trypsin

The activity of 30 μg of crystalline trypsin acting in 10 ml 0.01 *M* benzoyl arginine ethyl ester–HCl was measured at 30°. At pH 6 the inhibition by DNA, showing a maximum of 74 %, is much more pronounced than at pH 7.5. The inhibition curve at pH 6 displayed a sharp break similar to the case of ChT, and indicated a combination ratio of 7:1 by weight, provided the enzyme preparation did not contain inactive protein.

Some differences in nucleic acid–protein complexes extracted from tissues and those produced in the test tube can be expected, but no compelling evidence has been advanced that these combinations differ fundamentally (see ref. 4). In this connection it should be mentioned that a soluble complex, similar to a natural nucleoprotamine, is obtained when DNA and protamine are mixed in very dilute solutions[5]. It should also be noted that the protein component of tobacco mosaic virus recombines spontaneously with either the original nucleic acid to form the active virus[6] or with certain other nucleic acids of the RNA type to form inactive particles with a structure similar to that of the virus[7]. The protein: nucleic acid weight ratio of the chymotrypsin and trypsin complexes is of the same order as that of many RNA and DNA viruses[4]. Also, the accommodation of ChT on the DNA chain in a ratio of one protein molecule per four nucleotide units that together are of the order of one twentieth its size, might require considerable molecular orientation. If the structure of such complexes were similar to that of biologically active nucleoproteins, the latter could be investigated on an exact kinetic basis with the present systems serving as models. However, further studies are needed to determine whether this is the case.

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[1] E. CHARGAFF, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. I, Academic Press Inc., 1955.

[2] Symposium on Biocolloids, *J. Cellular Comp. Physiol.*, 49, Suppl. 1 (1957).

[3] *Cellular Biology of Nucleic acids and Viruses*, (T. M. RIVERS, consulting ed.) Vol. V, Special publication of New York Acad. Sci., 1957, p. 1–414.

[4] *Nucleoproteins*, 11th Chemistry Conference, Solvay Institute, Interscience Publ., 1959.

[5] P. ALEXANDER, *Biochim. Biophys. Acta*, 10 (1953) 595.

[6] H. FRAENKEL-CONRAT AND R. C. WILLIAMS, *Proc. Natl. Acad. Sci. U.S.*, 41 (1955) 690.

[7] R. G. HART AND J. D. SMITH, *Nature*, 178 (1956) 1739.

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The asymmetric acetylation of (±)-butan-2-ol by *p*-nitrophenyl acetate and α-chymotrypsin

BALLS, ALDRICH, McDONALD AND WOOD[1–3] have shown that α-chymotrypsin catalyzes a trans-esterification reaction between *p*-nitrophenyl acetate and a number of aliphatic alcohols. They were able to isolate ethyl acetate from an aqueous solution, buffered at pH 6.2, initially containing ethanol, *p*-nitrophenyl acetate and α-chymotrypsin and, by means of suitable analytical techniques to demonstrate the probable formation of alkyl acetates from other alcohols. In this latter study it was found that in general the apparent rate of acetylation increased with increased chain length of the normal alcohols and decreased with increased branching of the isomeric butanols.

Using techniques similar to those employed by McDONALD AND BALLS[3] it has now been shown that *p*-nitrophenyl acetate in the presence of either α- or δ-chymotrypsin reacts with (±)-butan-2-ol to give predominantly (—)-2-butyl acetate. We have confirmed the earlier finding that (—)-2-butyl acetate possesses the same configuration as (—)-butan-2-ol[4], which, alternatively, may be designated as (R)-butan-2-ol[5]. Thus, it is (R)-butan-2-ol that is preferentially acetylated by *p*-nitrophenyl acetate in the presence of α- or δ-chymotrypsin.

The preceding experiments afford a direct and unambiguous demonstration of the asymmetry of those portions of the active sites of α- and δ-chymotrypsin that are responsible for the enzyme-catalyzed solvolysis of *p*-nitrophenyl acetate. Furthermore, the knowledge that (R)-butan-2-ol is acetylated at a greater rate than (S)-butan-2-ol provides us with a new approach to the problem of definition of the topography of the active sites of these enzymes. It now appears possible to determine the stereochemical consequences of chymotrypsin-catalyzed trans-esterification reactions involving a variety of optically active alcohols, and possibly amines, including those containing a carboxyl group, or a derivative thereof. Such studies are in progress.

Experimental

To 500 ml of an aqueous solution 0.7 *M* in (±)-butan-2-ol and 0.7 *M* in the phosphate component of a NaH_2PO_4–Na_2HPO_4 buffer, pH 6.2, was added 0.45 g (2.28 mmole) of *p*-nitrophenyl acetate in 40 ml acetone and 250–400 mg of α- or δ-chymotrypsin in about 50 ml water. The reaction mixture was maintained at room temperature for 48 h, an additional 0.45 g *p*-nitrophenyl acetate added, and after 24 h the reaction mixture was extracted with ethyl ether (5 × 40 ml). The ethereal extracts were combined, dried over anhyd. $MgSO_4$ and the ether removed by distillation. Fractional distillation of the residue through a 30-cm column packed with glass helices gave approximately 15 ml butan-2-ol containing less than 1 % of 2-butyl acetate as judged by the intensity of a ferric hydroxamate test. Concentration of the ester was achieved by vapor-phase chromatography on a 0.635 × 152.4-cm Apiezon column at 65°. The distillate was introduced onto the column in 0.2-ml portions. The ester fractions, from all portions, were collected in a single trap, containing glass wool and cooled in liquid nitrogen, after the more rapidly moving butan-2-ol fractions had issued from the column. The contents of the trap containing the ester fractions were distilled from the trap to give 0.75–1.0 ml of a second distillate containing about 10 % of 2-butyl acetate in butan-2-ol, the exact composition being determined by an independent vapor-phase chromatographic analysis. The optical rotation of the distillate was then determined and the relative amounts of (R)- and (S)-butan-2-ol calculated on the basis of a value of $[\mathrm{M}]_{\mathrm{D}}^{52} = -30^\circ$ for (R)-butan-2-ol[6]. The results obtained from three experiments are summarized in Table I. No 2-butyl acetate was produced when the experiment was conducted in the absence of enzyme and no trans-esterification was detected during distillation of butan-2-ol in the presence of *p*-nitrophenyl acetate. The configurational relationship between (—)-2-butyl acetate and (—)-butan-2-ol was checked by esterification of (—)-butan-2-ol, prepared by the method of INGERSOLL[7], with acetic anhydride. No inversion of the sign of rotation was observed.

TABLE I

PROPERTIES OF ESTER FRACTION

Expt.	*% Ester (W/W)**	*α_D***	*(R)-butan-2-ol %*
1	2.84	— 0.38	82
2	7.75	— 0.78	75
3	10.85	— 1.55	86

* Density taken as 0.80.
** Rotation observed in 1-dcm tube at 25°.

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[1] A. K. BALLS AND F. L. ALDRICH, *Proc. Natl. Acad. Sci. U.S.*, 41 (1955) 190.
[2] A. K. BALLS AND H. N. WOOD, *J. Biol. Chem.*, 219 (1956) 245.
[3] C. E. MCDONALD AND A. K. BALLS, *J. Biol. Chem.*, 221 (1956) 993.
[4] W. KLYNE, *Progr. in Stereochem.*, 1 (1954) 182.
[5] R. S. CAHN, C. K. INGOLD AND V. PRELOG, *Experientia*, 12 (1956) 81.
[6] W. KLYNE, in E. A. BRAUDE AND F. C. NACHOD, *Determination of Organic Structures by Physical Methods*, Academic Press, New York, 1955, p. 84.
[7] A. W. INGERSOLL, *Organic Reactions*, 2 (1944) 403.

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Formation of the cyclic acetal phospholipid during alkaline and enzymic hydrolysis of choline plasmalogen

FEULGEN AND BERSIN[1] and THANNHAUSER *et al.*[2] isolated what was apparently a cyclic acetal phospholipid from the alkaline-hydrolysis products of muscle and brain phospholipids respectively. It is, however, now generally accepted that the natural aldehydogenic phospholipids (plasmalogens) are derivatives of glycerylphosphorylcholine, ethanolamine or -serine with two fatty residues, one a fatty acid and the other an acid-labile aldehydogenic residue linked to the glycerol moiety as a $\alpha\beta$-unsaturated ether[3–5]. Lysoplasmalogens possessing this $\alpha\beta$-unsaturated ether structure, determined by the iodine method of SIGGIA AND EDSBERG[6], have been isolated from ethanolamine plasmalogen[7] and choline plasmalogen[8] by removal of the acyl group with alkali. The origin and nature of Feulgen's cyclic acetal phospholipid is not clear, and it has been suggested that the identification was mistaken. Preliminary work in this laboratory showed, however, that on hydrolysis of choline plasmalogen either by snake venom[4] or by alkali there was an apparent loss of total aldehyde, as measured by a standard colorimetric technique of hydrolysis in 90 % acetic acid at 50° and condensation with Feulgen reagent[9], which was in fact due largely to formation of some acid-stable derivative.

The course of decomposition of choline plasmalogen was therefore examined using three methods for estimation of aldehyde: (A) hydrolysis in 90 % acetic acid at 50°, (B) hydrolysis in 90 % acetic acid containing 0.5 N HCl and 0.0015 N $HgCl_2$ at 50°; this was followed in both cases by condensation with Feulgen reagent for 20 min at 0°. (C) hydrolysis in the acetic acid–HCl–$HgCl_2$ reagent at 0° in presence of the Feulgen reagent for 20 min.

The material used was a "lecithin" fraction of ox-heart muscle containing about equal proportions of choline plasmalogen and phosphatidyl choline; the aldehyde value (taken as 100 %) was the same by methods A and B. On treatment of a portion with *Agkistrodon piscivorus* venom (phospholipase A), the aldehyde value by method A decreased gradually with the decrease in fatty acid ester and reached a constant value of 80 % of the original, but the value by method B remained at 100 %.

On saponification of another portion in 0.1 N methanolic NaOH at 38° for 15 min the aldehyde value fell to 70 % by method A and to 90 % by method B (the latter result possibly indicating that about 10 % of the aldehyde was split off and polymerised). The aldehydogenic material (molar ratio P:N, 1:1) was recovered quantitatively by chromatography on silicic acid and cellulose columns; it had a molar ratio of aldehyde: P of 0.75:1 by method A and 0.94:1 by method B, and an $\alpha\beta$-unsaturated ether: P ratio of 0.71:1 by the iodine method. It was concluded that the material was a mixture of the (straight-chain) lysoplasmalogen and a presumably cyclic form. A small amount of the lysoplasmalogen was obtained by fractionation on silicic acid, but the two forms could not be separated quantitatively. The more acid-stable form was therefore isolated by decomposition of the lysoplasmalogen in acetic acid; it was contaminated with some substance, not yet identified, which was detected by chromatography on silicic-impregnated paper. This substance contained phosphorus and choline but no aldehyde and is possibly a glyceryl ether. The pure cyclic form was obtained by further fractionation on alumina.

The rates of liberation of aldehyde from choline plasmalogen, the lysoplasmalogen, the cyclic form and the standard dimethyl acetal of palmitaldehyde by method A (Fig. 1) and the aldehyde values for the different compounds obtained by methods A, B and C (Table I) show that two compounds are formed from choline plasmalogen on saponification with alkali or by treatment with snake venom; a lysoplasmalogen with an $\alpha\beta$-unsaturated ether structure, which is more labile to acid than the original plasmalogen, and a cyclic form which is more stable to acid than either the original plasmalogen or the standard dimethyl acetal of palmitaldehyde. This explains some recent observations[10, 11] on the products of alkaline hydrolysis of plasmalogens.

We wish to acknowledge the support of this work by the British Empire Cancer Campaign.

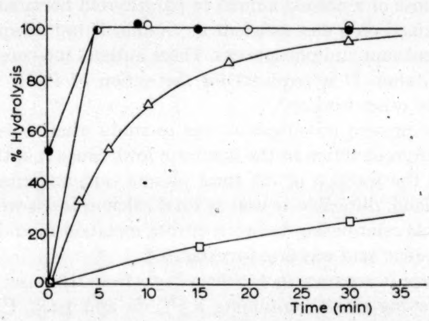

Fig. 1. The course of liberation of aldehyde from intact plasmalogen (△); straight-chain lysoplasmalogen (●); cyclic form (□); dimethyl acetal of palmitaldehyde (○); by method A.

TABLE I

RELATIVE ALDEHYDE VALUES BY DIFFERENT METHODS OF ESTIMATION

Compound	*Aldehyde values by method*		
	A	*B*	*C*
Dimethyl acetal of palmitaldehyde	100	100	68
Choline plasmalogen	100	100	68
Lysoplasmalogen	100	100	100
Cyclic form	33	100	3–5

A. 45 min 90 % acetic acid at 50°; 20 min + Feulgen reagent at 0°.
B. 5 min in acetic–HCl–$HgCl_2$ at 50°; 20 min + Feulgen reagent at 0°.
C. 20 min at 0° acetic–HCl–$HgCl_2$ in presence of Feulgen reagent.

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[1] R. FEULGEN AND T. BERSIN, *Z. physiol. Chem.*, 260 (1939) 217.
[2] S. Y. THANNHAUSER, N. F. BONCODDO AND G. SCHMIDT, *J. Biol. Chem.*, 188 (1951) 417.
[3] E. KLENK AND H. DEBUCH, *Z. physiol. Chem.*, 296 (1954) 179.
[4] M. M. RAPPORT AND R. E. FRANZL, *J. Biol. Chem.*, 225 (1957) 851.
[5] M. M. RAPPORT AND R. E. FRANZL, *J. Neurochem.*, 1 (1957) 303.
[6] S. SIGGIA AND R. L. EDSBERG, *Anal. Chem.*, 20 (1948) 762.
[7] M. M. RAPPORT, B. LERNER, N. ALONZO AND R. FRANZL, *J. Biol. Chem.*, 225 (1957) 859.
[8] E. F. HARTREE AND T. MANN, *Biochem. J.*, 75 (1960) 251.
[9] G. M. GRAY AND M. G. MACFARLANE, *Biochem. J.*, 70 (1958) 409.
[10] R. M. C. DAWSON, *Biochem. J.*, 75 (1960) 45.
[11] G. SCHMIDT, B. OTTENSTEIN, W. A. SPENCER, S. KECK, R. BLIETZ, Y. PAPAS, D. PORTER, M. L. LEVIN AND S. Y. THANNHAUSER, *Am. J. Diseases Children*, 97 (1959) 691.

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On the interaction between vitamin D and parathyroid hormone in the domestic fowl

It has recently been reported by HARRISON, HARRISON AND PARK[1] that the typical hypercalcaemic response of a normal animal to parathyroid hormone does not occur in young rats maintained on a diet deficient in vitamin D but adequate in all other nutrients, including calcium and phosphorus. These authors interpreted their results as indicating that vitamin D is required for the action of PTH. Similar findings have been reported by other workers[2].

The object of the present investigation was to study whether or not vitamin D is necessary for parathyroid action in the domestic fowl. Since it is the ionic calcium which is regarded as the fraction of the total plasma calcium primarily influenced by the parathyroid gland, diffusible as well as total calcium levels were studied, and, in view of the probable relationship between citrate metabolism and vitamin D and PTH action, plasma citric acid was also investigated.

The birds were kept in separate metabolism cages from the onset of reproduction and fed a standard laying ration containing 2.3 % Ca and 1.1 % P. The basal diet was virtually devoid of vitamin D, though adequate in all other respects, and the control birds received this diet supplemented with vitamin D_3 at a level of 200 i.u./100 g diet. As a result of the deficient diet, the shells of the eggs laid by the birds on the experimental diet became progressively thinner and the rate of lay declined, until after 6 months egg production ceased almost entirely, with the birds laying 1 egg/month on the average. Virtual cessation of lay was in fact the criterion used to indicate that a serious deficiency of vitamin D had been induced.

Approx. 10 ml blood (providing about 7.0 ml plasma) were required for each set of determinations, and since it was not desirable to withdraw two samples of this magnitude from a hen within a few hours, the first sample was taken 24 h before injection of PTH. However, a small sample of blood (0.5 ml) was taken immediately prior to PTH injection to check that the level of total calcium had not altered materially since the previous day. In the hens deficient in vitamin D no changes were observed but small differences were found in some of the control birds. Previous experiments (unpublished) had shown that the diffusible calcium of the plasma of laying hens is very constant from day to day at any particular stage of the laying cycle, and the level of total calcium just before PTH injection, rather than that observed the previous day, was used to compare with the level after injection. The control birds were bled from 1–4 h after oviposition, *i.e.*, when egg-shell calcification was not in progress.

PTH ("Para-thor-mone", Lilly) was injected subcutaneously at a rate of 75 units/kg live weight and the blood samples were taken 2–2.5 h afterwards, when the maximum response to the hormone is observed (unpublished observations). A plasma ultrafiltrate, on which diffusible calcium was determined, was obtained using the apparatus described by ROSE[3]. The pH of the plasma was controlled throughout the ultrafiltration by circulating a mixture of 5 % CO_2 in oxygen around the collodion filtration shell. Determinations of total and diffusible calcium were made by a photometric titration with EDTA at pH 12–13 using murexide as indicator,

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Abbreviations: PTH, parathyroid hormone; EDTA, ethylenediaminetetraacetate.

TABLE I

EFFECT OF PARATHYROID EXTRACT ON THE MEAN CONCENTRATIONS OF TOTAL, DIFFUSIBLE AND NON-DIFFUSIBLE CALCIUM AND CITRIC ACID OF THE PLASMA OF VITAMIN-D-DEFICIENT AND NORMAL HENS

Values are means (in mg/100 ml) and standard errors of mean.

Treatment	*No. of animals*	*Total*		*Diffusible*		*Non-diffusible*		*Citric acid*	
		Before PTH	*After PTH*	*Before PTH*	*After PTH*	*Before PTH*	*After PTH*	*Before PTH*	*After PTH*
Vitamin D deficien	7	13.4 ± 0.7	13.4 ± 0.7 (N.S.)	6.4 ± 0.2	7.6 ± 0.3*	6.9 ± 1.0	5.8 ± 0.7*	3.37 ± 0.44***	3.91 ± 0.44*** (N.S.)
Control (laying)	7	22.7 ± 1.7	26.1 ± 3.3**	8.2 ± 0.4	10.1 ± 0.8*	14.5 ± 1.4	16.0 ± 2.4 (N.S.)	2.64 ± 0.12	2.58 ± 0.18 (N.S.)

* Significantly different ($P < 0.05$) from the level before PTH injection.
** Significantly different ($P < 0.01$) from the level before PTH injection.
*** Data available for 4 birds only.
N.S. Not significantly different from the level before PTH injection.

and citric acid was determined on trichloroacetic acid filtrates of plasma by the method of TAYLOR[4].

The levels of citric acid, total, diffusible and non-diffusible calcium in the plasma after PTH injection were compared statistically with the corresponding levels before injection, using the paired "*t*" test, and the results are shown in Table I. In the control group, PTH exerted a typical effect on the blood calcium, elevating both total and diffusible levels significantly, though the increase in the non-diffusible calcium was not significant. The mean plasma citric acid level was not affected by the PTH and it seems probable that the high level of oestrogen in the circulation prevented the expected rise.

PTH treatment had no effect on the total calcium level of the plasma of the birds deficient in vitamin D. The diffusible calcium and the citric acid levels on the other hand were increased (significantly only in the former), and it would appear, therefore, that vitamin D is not necessary for the action of PTH in the hen. Associated with the rise in diffusible calcium was a significant fall in the non-diffusible calcium level after PTH injection. It seems most unlikely that this can have been due to a reduction in the protein concentration of the plasma since the main bleedings were only 24 h apart and a fall in total calcium would have been expected at the time of the bleeding immediately before the PTH injection if a fall in plasma proteins had occurred. LLOYD AND ROSE[5], as a result of investigations on 12 hyperparathyroid patients before and after operation, and on a normal subject treated with PTH, have produced evidence that PTH reduces the calcium-binding power of the plasma proteins, a conclusion also reached by FANCONI AND ROSE[6]. The results of the present experiments are consistent with this suggestion.

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[1] H. C. HARRISON, H. E. HARRISON AND E. A. PARK, *Am. J. Physiol.*, 192 (1958) 432.
[2] C. MARNAY AND Y. RAOUL, *Comp. rend. soc. biol.*, 153 (1959) 1949.
[3] G. A. ROSE, *Clin. Chim. Acta*, 2 (1957) 227.
[4] T. G. TAYLOR, *Biochem. J.*, 54 (1953) 48.
[5] H. M. LLOYD AND G. A. ROSE, *Lancet*, (1958 II), 1258.
[6] A. FANCONI AND G. A. ROSE, *Quart. J. Med.*, 27 (1958) 463.

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Cytochrome 556 and electron-transport system in snail hepatopancreas

The occurrence of a cytochrome-like haemoprotein called enterochrome 556 in the gut-fluid of garden snails has recently been reported and some of its properties have been described[1]. A cytochrome resembling enterochrome 556 has now been found to occur in the hepatopancreatic cells of garden snails, *Euhadra amaliae* and *E. sandai*.

Abbreviation: DPNH, reduced diphosphopyridine nucleotide.

This cytochrome is similar to cytochrome $h^{2,3}$ in certain respects, but definitely differs from it in several characteristics. It has, therefore, been provisionally designated as "Cytochrome 556".

The cytochrome was partially purified from the tissues of snail hepatopancreas as follows. Excised tissues were rinsed with snail Ringer solution[4], and stored at — 15° until needed. The frozen tissues were homogenized with 3 vol. water in a Waring blendor, passed through a thin cloth, and centrifuged. The supernatant collected was treated with a small amount of 0.5 *M* lead acetate, followed by the procedures applied for the purification of enterochrome 556[1]. The preparation of cytochrome 556 thus obtained was a clear orange-red solution.

The visible absorption maxima of reduced cytochrome lie at 556 mμ (α), 526 mμ (β), and 424 mμ (Soret; 412 mμ, oxidized form). The peaks in the pyridine haemochromogen spectrum are located at 553 mμ (α), 521 mμ (β), and 415 mμ (Soret). The oxidation–reduction potential as measured with the aid of 2,6-dichlorophenol indophenol was found to be approximately + 0.20 V at pH 7.0, 30°. The prosthetic group could be easily split off from the protein moiety by the treatment with acetone containing 1 % HCl, and the acid haematin thus separated was soluble in ether. The cytochrome was found to combine neither with CO nor with HCN at pH 7.0, and to show no peroxidase activity. It migrated toward the cathode when subjected to paper electrophoresis in 0.05 *M* phosphate buffer, pH 7.0. These properties of cytochrome 556 are almost identical with those of enterochrome 556[1].

Spectroscopic examinations have shown that cytochrome 556 is abundantly present both in mitochondrial and microsomal fractions of snail hepatopancreas. Cell particulates were prepared by differential centrifugation from tissue homogenates in 0.2 *M* sucrose. After removal of nuclei and cell debris at 600 × *g* for 10 min, mitochondria were sedimented at 9,000 × *g* for 15 min. Microsomes were sedimented at 78,000 × *g* for 60 min as firmly packed pellets. Cytochrome contents were estimated from difference spectra by applying μ*M* extinction coefficients recommended by CHANCE[5]. The coefficient for cytochrome 556 was assumed to be equal to that of cytochrome *b*. The mitochondrial fraction contained, in addition to cytochrome 556 (0.25 μmole/g protein), significant quantities of cytochromes *a* and *b* (0.19 and 0.20 μmole/g protein, respectively), but cytochrome *c* could not be detected even at liquid–air temperature. The microsomal fraction, on the other hand, contained cytochrome 556 (0.24 μmole/g protein) as the only detectable cytochrome component. The fact that cytochrome 556 is richly localized in hepatopancreatic particulate structures and that this organ has a remarkable secreting activity strongly supports the view that enterochrome 556 is a secreted form of cytochrome 556.

Hepatopancreatic mitochondria were able to catalyze the aerobic oxidation of reduced cytochrome 556 as well as of reduced enterochrome 556, whereas these haemoproteins could be rapidly reduced by DPNH under anaerobic conditions in the presence of either mitochondria or microsomes.

As illustrated in Fig. 1, the addition of DPNH to the aerobic suspension of mitochondria caused the immediate reduction of cytochrome 556. On exhaustion of O_2, the peaks of cytochrome 556 were intensified and those of reduced cytochrome *a* appeared simultaneously. When succinate was added to aerobic mitochondria, only slight peaks due to reduced cytochrome *b* were observed. This spectrum changed to that observed in the case of DPNH addition after the complete exhaustion of O_2.

The DPNH-cytochrome *c* reductase activity of the mitochondria was not inhibited by 2–10 μg/ml antimycin A, while the succinate-cytochrome *c* reductase activity was completely inhibited by 2 μg/ml antimycin A.

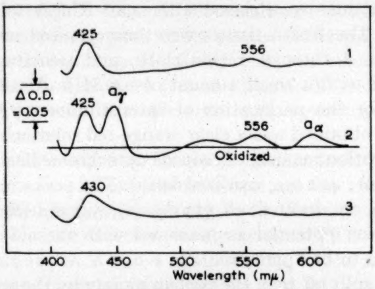

Fig. 1. Difference spectra of the cytochrome components of mitochondria prepared from hepatopancreas, suspended in 0.05 *M* phosphate buffer, pH 7.0. Light path of cuvettes, 1 cm; concn., 5 mg protein/ml. Spectra were recorded with a Cary model 14 recording spectrophotometer. Curve 1: Difference between DPNH-added aerobic mitochondria and aerobic mitochondria. Curve 2: Difference between DPNH-reduced anaerobic mitochondria and aerobic mitochondria. Curve 3: Difference between succinate-added aerobic mitochondria and aerobic mitochondria.

From these experimental results the following scheme may be suggested as a possible pathway of electron transport in the mitochondria of snail hepatopancreas.

DPNH ⟶ Flavoprotein ↘
Cyt. 556 ⟶ Cyt. *a* (oxidase) ⟶ O_2
↑
Factor (antimycin A-sensitive)
↑
Cyt. *b*
Succinate ⟶ Succinic dehydrogenase ↗

As may be seen, cytochrome 556 appears to play a role similar to that of cytochrome *c* in the electron-transport system in mammalian mitochondria.

Full experimental details will be published elsewhere. The author wishes to thank Dr. RYO SATO for his encouragement and advice in this investigation.

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[1] K. KAWAI, *Biochim. Biophys. Acta*, 43 (1960) 349.
[2] J. KEILIN, *Biochem. J.*, 64 (1956) 663.
[3] J. KEILIN, *Nature*, 180 (1957) 427.
[4] E. BALDWIN, *Biochem. J.*, 29 (1935) 1538.
[5] B. CHANCE, *Nature*, 169 (1952) 215.

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Formation of D-ribulose from D-gluconate in guinea-pig liver

Recent studies[1] in this laboratory on the substrate specificity of DPN–L-gulonic acid dehydrogenase from guinea-pig liver revealed that D-gluconic acid was also dehydrogenated, as shown by the increase of absorbancy at 340 mμ. D-Gluconic acid might easily be produced *in vivo* from glucose by D-glucose dehydrogenase in liver or by other enzyme systems. SMILEY AND ASHWELL[2] also reported that a similar enzyme dehydrogenates the aldonic acids with L-hydroxy group at the third carbon atom. The present paper deals with the reaction products from D-gluconic acid by this dehydrogenation using uniformly labeled [^{14}C]gluconic acid.

The enzyme was purified by the method reported by ISHIKAWA[3] in this laboratory. [^{14}C]Gluconic acid uniformly labeled was obtained by electrolytic oxidation with NaBr from uniformly labeled [^{14}C]glucose (0.1 μC/μmole) and purified with Dowex-1. The reaction was performed in double-side-arm Warburg flasks as described in the legend to Fig. 1. After a 2-h incubation at 37°, CO_2 was absorbed in KOH after addition of trichloroacetic acid, and precipitated as $BaCO_3$. Decarboxylation, determined with a Q-gas counter, amounted to 894.1 ± 5.6 counts/min (infinite thickness) in the first experiment, 1004.6 ± 5.9 counts/min in the next under the same conditions, while nonenzymically 66.8 ± 2.0 counts/min were recorded. The remaining carbon chain was identified as ribulose as follows. The 10 mg of ribulose syrup obtained from epimerization of D-arabinose[4], containing D-ribose and D-arabinose in smaller amounts, was added as carrier to the deproteinized mixture. After the mixture was desalted and concentrated, it was dissolved in 0.005 M $Na_2B_4O_7$

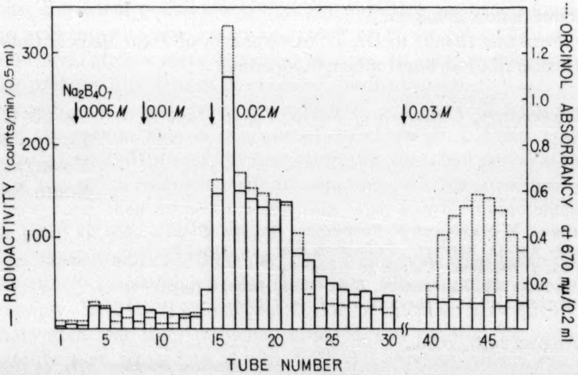

Fig. 1. Elution curve of the reaction product. The reaction mixture contained: 15 μmoles (1.5 μC) sodium gluconate in side-arm I; 30 μmoles DPN, 100 μmoles nicotinamide, 0.2 μmole $MnCl_2$, 0.8 ml 0.1 M glycine buffer, pH 10.5, and 1.0 ml of the enzyme solution in main chamber; 50 % trichloroacetic acid in side-arm II; and 0.2 ml conc. KOH in central well; total volume, 3.2 ml. A Dowex-1-borate 0.9 × 12.5 cm column was used for the fractionation and the volume of each fraction was 10 ml. Radioactivity is expressed in solid lines as counts/min/0.5 ml. Absorbancy at 670 mμ is expressed in dotted lines as estimated after 0.2 ml of the fraction was reacted with orcinol and diluted to 4.0 ml.

Abbreviation: DPN, diphosphopyridine nucleotide.

and analysed on a Dowex-1-borate column as shown in Fig. 1. Radioactivity was concentrated in the first orcinol-positive peak. Experiments with non-labeled gluconate (100 μmoles in an otherwise identical experiment) gave rise to an orcinol-positive peak in the same position. These peaks were concentrated after being passed through a Dowex-50-hydrogen column, and boric acid was removed as the methanol complex. The resulting syrup showed a violet color by spot test with anthrone, and its orcinol reaction showed a ratio of absorbancy at 670 mμ to that at 540 mμ[5], which suggested the presence of keto-pentose. The autoradiograph of the syrup thus obtained showed the same R_F value as that of ribulose. The product was also subjected to reaction with *o*-nitrophenylhydrazine according to GLATTHAAR's procedure[4]. The resulting hydrazone was fractionated on an alumina column and its radioactivity was counted. The first yellow band eluted with ethyl acetate was *o*-nitrophenylhydrazine. Following that, elution by acetone, *n*-propanol, ethanol, and methanol produced peaks but no radioactivity was detected in them. Radioactivity was eluted with methanol–water (1:1), and this orange peak appeared in the same position as authentic ribulose *o*-nitrophenylhydrazone (m.p. 168°). 10 mg of ribulose *o*-nitrophenylhydrazone was added to the eluate and the specific activity of the crystal was determined during 5 recrystallizations. The weight of the hydrazone was estimated from the molar extinction coefficient ($6.0 \cdot 10^3$) at 432 mμ, and the samples were counted in the state of infinite thinness. The specific radioactivity was found to be almost constant during the recrystallization as follows. 1, 170.1 ± 17.4; 2, 138.5 ± 9.1; 3, 166.0 ± 11.9; 4, 158.3 ± 10.2; 5, 180.9 ± 13.4 counts/min/mg. This enzyme also dehydrogenated 6-phosphgluconate[5] slowly, but slight alkaline phosphatase activity was also detected in the preparations used. Further purification of this enzyme is now going on.

We express our thanks to Dr. I. YANAGISAWA of Toho Medical College for the gift of authentic ribulose *o*-nitrophenylhydrazone.

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[1] Y. MACHIYAMA, Y. MANO AND N. SHIMAZONO, *Biochim. Biophys. Acta*, 42 (1960) 564.
[2] J. D. SMILEY AND G. ASHWELL, *Federation Proc.*, 19 (1960) 85.
[3] S. ISHIKAWA, *J. Biochem. (Tokyo)*, 46 (1959) 347.
[4] C. GLATTHAAR AND T. REICHSTEIN, *Helv. Chim. Acta*, 18 (1935) 80.
[5] B. L. HORECKER AND P. Z. SMYRNIOTIS, *J. Biol. Chem.*, 193 (1951) 371.

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Selective formation of α-amylase by non-growing cells of *Pseudomonas saccharophila*

Several years ago, RICKENBERG AND LESTER[1] reported that a mutant of *Escherichia coli* produced 5 % as much β-galactosidase when induced under certain non-growing conditions as was produced during logarithmic growth. They suggested that the enzyme formed in non-growing cells indicated preferential synthesis of β-galactosidase in relation to other cellular proteins. Subsequent investigations by MANDELSTAM[2] and others[3] have demonstrated that a significant fraction (5–6 %/h) of the proteins of non-growing cells of *E. coli* undergoes turnover, and as a result of these studies, it was presumed that the observations of RICKENBERG AND LESTER could still be explained on the basis of a constant "differential rate of synthesis" of β-galactosidase[4]. Similar assumptions could be made on analogous inducible systems in resting yeast cells[5,6].

Recently we reported that *Pseudomonas saccharophila* forms an extracellular α-amylase in phosphate buffer when incubated with starch under conditions where there is no detectable increase in protein. Furthermore, in periods of up to 4 h, such cells produce as much enzyme as do comparable quantities of growing cells[7]. It was also found that this enzyme is formed at a differential rate of 1:1200 in cells growing with starch as the carbon source[8], *i.e.*, α-amylase represents 0.08 % of the total proteins synthesized.

It seemed obvious that α-amylase, if it is formed *de novo*, is not being made at the same differential rate with respect to other proteins in resting cell suspensions as in growing cultures. Evidence has already been presented[8] in this connection, supporting the view that this enzyme is not formed from high-molecular-weight precursors, but rather *de novo*. On the basis of these observations, it seemed certain that the formation of α-amylase in resting cells represents a selective (preferential) synthesis of this protein.

It is the purpose of this communication to give details regarding this contention and to measure the extent of this selectivity. The objective was to introduce a radioactive amino acid into the free amino acid pool[9] of *P. saccharophila* simultaneous with induction and, after a suitable period of time, to compare the radioactivity of the cellular proteins with that of the isolated, purified enzyme.

Growth conditions, requirements for induction, and methods for isolation and purification of the enzyme have been reported elsewhere[10,11]. Labeled aspartate or glutamine were introduced into cells by first subjecting them to a period of starvation (aeration for 4 h at 30° in complete medium, containing 0.2 % sucrose but without a nitrogen source), and then suspending the cells with 0.01 % (final concn.) of the labeled compound in phosphate buffer (0.033 M, pH 6.8) plus starch (0.2 % final concn.). Under these conditions, these particular compounds were taken up into the pool more effectively than without prior starvation. When labeled methionine was used, it was simply added (0.03 % final concn.) during induction in buffer. In all cases, cells were harvested and their proteins extracted and counted for radioactivity. The supernatants were treated to obtain purified α-amylase, which was similarly counted.

The results (Table I) reveal that the ratio of labeled amino acid incorporated into enzyme to that into cellular protein is approximately 1:40. An experiment in which cells were grown on starch in the presence of radioactive aspartate is also included and it is evident that the incorporation ratio thus obtained is compatible with the finding that α-amylase represents 0.08 % of the total protein in such cultures.

The results substantiate the contention that α-amylase is selectively formed in non-growing cells of *P. saccharophila*. In contrast to other cases in which preferential synthesis of specific enzymes has been shown[12,13], this case is not accountable for on

TABLE I

INCORPORATION OF LABELED AMINO ACIDS INTO CELLULAR AND α-AMYLASE PROTEINS

Amino acid used	*Medium*	*Incubation period* (min)*	*Total radioactivity*		*Incorporation ratio***
			Amylase counts/min × 10^{-2}	*Cell protein counts/min × 10^{-2}*	
[^{14}C]-L-aspartic	Buffer	90	300	10400	35
[^{14}C]-L-glutamine	Buffer	90	35.7	1710	48
[^{35}S]-methionine	Buffer	180	13.0	569	44
[^{14}C]-L-aspartic	Complete	18 h	30.3	31400	1040

* Cells induced in buffer showed no increase in total proteins during the periods of incubation. Cells grown in the presence of labeled aspartic acid were derived from a small inoculum and allowed to reach a turbidity which measured 400 in the Klett colorimeter at 540 mμ. At this time, the cells were in the late logarithmic phase.

** Incorporation ratio = counts/min in cellular proteins: counts/min in α-amylase.

the basis of a relief from repression[14]. In this system, the absolute amounts of amylase protein formed by growing and non-growing cells in short-term experiments are comparable. Thus, the selectivity here concerns not the increased rate of formation of one enzyme against the background of a constant synthesis of other cellular proteins, but rather the decreased rate of formation of all cellular proteins against the constant synthesis of α-amylase. These bacteria therefore appear to possess a control mechanism of a more generalized nature than others that have been described[15].

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[1] H. V. RICKENBERG AND G. LESTER, *J. Gen. Microbiol.*, 13 (1955) 279.
[2] J. MANDELSTAM, *Biochem. J.*, 69 (1958) 110.
[3] E. BOREK, L. PONTICORVO AND D. RITTENBERG, *Proc. Natl. Acad. Sci.*, 44 (1958) 369.
[4] J. MONOD, G. COHEN-BAZIRE AND M. COHN, *Biochim. Biophys. Acta*, 1 (1951) 585.
[5] M. STEPHENSON AND J. YUDKIN, *Biochem. J.*, 30 (1936) 506.
[6] S. SPIEGELMAN AND R. DUNN, *J. Gen. Physiol.*, 31 (1948) 153.
[7] J. A. SCHIFF, J. M. EISENSTADT AND H. P. KLEIN, *J. Bacteriol.*, 78 (1959) 124.
[8] J. M. EISENSTADT AND H. P. KLEIN, *J. Bacteriol.*, 77 (1959) 661.
[9] A. MARKOVITZ AND H. P. KLEIN, *J. Bacteriol.*, 70 (1955) 649.
[10] A. MARKOVITZ AND H. P. KLEIN, *J. Bacteriol.*, 70 (1955) 641.
[11] A. MARKOVITZ, H. P. KLEIN AND E. H. FISCHER, *Biochim. Biophys. Acta*, 19 (1956) 267.
[12] L. GORINI AND W. MAAS, in W. D. MCELROY AND B. GLASS, *Chemical Basis of Development*, Johns Hopkins Press, Baltimore, 1958.
[13] A. TORRIANI, *Biochim. Biophys. Acta*, 38 (1960) 460.
[14] H. J. VOGEL, *Handbuch der Pflanzenphysiologie*, 1959, p. 117.
[15] B. MAGASANIK, A. K. MAGASANIK AND F. C. NEIDHARDT, (Ciba Symposium) *Relation of Cell Metabolism*, Little, Brown & Co., Boston, 1959, p. 334.

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STUDIES ON THE NUCLEOPROTEINS FROM HUMAN VIRULENT TUBERCLE *BACILLI* AND ATTENUATED BOVINE TUBERCLE *BACILLI* (BCG)

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SUMMARY

Nucleoproteins were extracted from bovine tubercle *bacilli* (BCG) and a human virulent strain of tubercle *bacilli*, E9656. These preparations could be separated by zone electrophoresis in glass beads into two RNAP zones one of which coincided with that of the DNAP. The two nucleoproteins cross-reacted in the OUCHTERLONEY[12] diffusion technique using a horse antiserum to E9656 human strain tubercle *bacillus**.

INTRODUCTION

The presence of nucleic acids in tuberculin (PPD) from human virulent tubercle *bacilli* was demonstrated by SPIEGEL-ADOLF AND SEIBERT[1]. Later nucleic acids were identified in the culture medium by electrophoretic separation[2], and the same authors showed that considerable amounts of DNA were present. Nucleic acids were also isolated from the tubercle *bacillus* by various workers[3,4].

However, it was not until 1949 that any detailed chemical studies on mycobacterial nucleoproteins were carried out. CHARGAFF AND SAIDEL[5], using an avian strain of tubercle *bacilli* isolated a purified nucleoprotein which was mainly of the DNA type although RNA was also identified in the crude nucleoprotein. TSUMITA AND CHARGAFF[6] have recently published an article on the composition of the DNA and the DNAP from bovine tubercle *bacilli* (BCG). This work is mainly concerned with the isolation of DNAP and the study of the preparation, composition, and nucleotide arrangement of the nucleic acid and the investigation of the protein moiety.

The aim of the present work was to investigate the possibility of obtaining nucleoproteins from virulent human tubercle *bacilli* and bovine tubercle *bacilli* (BCG), with a view to employing them as antigens. The experiments described here concern the extraction, composition, and some physico-chemical properties of these nucleoproteins.

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* Abbreviations: DNA, desoxyribonucleic acid; DNAP, desoxyribonucleoprotein; RNA, ribonucleic acid; RNAP, ribonucleoprotein.

MATERIALS AND METHODS

Growth of bacilli

Bacillus Calmette-Guerin (BCG)[*] and tubercle *bacilli* (E9656) virulent human strain[**] were grown for 3 weeks on Sauton medium at 37°. At the end of this period the *bacilli* were killed by adding 2 % phenol and harvested after 2 days; finally they were well washed with distilled water.

Isolation of nucleoprotein

All extractions were carried out at about +2°. 3 portions of 30 g wet weight *bacilli* were triturated in a mortar with 60–70 g pyrex glass powder grade 4 and a small amount of distilled water to make a paste. After about 10 min trituration more water was added to make a suspension and the mixture was centrifuged at 700 × *g* for 30 min at +2°. The supernatant was removed and the residue washed once with distilled water, the second supernatant being recovered after re-centrifugation. The pooled supernatants were centrifuged at 10,000 × *g* at room temperature to remove bacterial debris and then filtered through a Membrane filter No. 5[***] overnight at +4°. Most of the nucleoproteins were retained on the filter, whilst most of the proteins, polysaccharides, and low molecular weight compounds were found in the filtrate. The residue on the filter was dissolved in water and centrifuged at 31,000 × *g* to remove glycogen[5]. The supernatant obtained after centrifugation was dialyzed for at least 24 h against distilled water containing merthiolate before lyophilization. The resulting powder constituted the crude nucleoprotein. An attempt was made to purify these nucleoproteins according to Chargaff and Saidel[5], who observed that the conjugated protein was not precipitated by half-saturation with ammonium sulphate. The same volume of saturated ammonium sulphate was added to a solution of the crude nucleoproteins at pH 7 with stirring. The mixture was kept overnight at +4° and then centrifuged at 10,000 × *g* for 20 min. The crude BCG nucleoprotein yielded a fairly heavy precipitate which was shown to be rich in lipids; this was demonstrated by staining with sudan black. The supernatant still remained rather turbid even after repeated centrifugation. The E9656 solution also yielded a precipitate with ammonium sulphate which contained lipid, although not a very large amount, and the supernatant was only slightly turbid. The supernatants were dialysed against distilled water until there was no more ammonium sulphate present, and then lyophilized. The crude and "purified" nucleoproteins were then investigated chemically, by zone electrophoresis, ultracentrifugation, and agar diffusion.

Chemical analysis

Protein, RNA, and DNA were estimated by the Folin or biuret[7,7a] orcinol[8] and diphenylamine[9] methods respectively. Phosphorus was determined according to Allen[10] and nitrogen by the micro-Kjeldahl method[11].

Ultracentrifugal analysis

These analyses were kindly carried out by Engineer A. Birch-Andersen and

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[*] BCG *bacilli* were obtained from Dr. K. Tolderlund, State Serum Institute, Copenhagen, Denmark.

[**] The BCG and E9656 *bacilli* were grown on Sauton medium in the Tuberculin Department of State Serum Institute: Head of Department, Engineer M. Magnusson.

[***] Obtained from Membranefilter Gesellschaft, Sartorius-Werke, Göttingen, Germany.

Mr. CH. LARSEN of the State Serum Institute in the analytical Spinco Model E. The nucleoproteins were dissolved in phosphate buffer pH 7.3 to give a 0.6 % solution.

Agar-diffusion was carried out according to the technique of OUCHTERLONEY[12].

Zone electrophoresis of the nucleoproteins

Separation of the nucleoproteins was first attempted using starch and starch-gel as supporting media. Neither of these two materials was suitable since the nucleoproteins did not migrate in these carriers, but the DNA, RNA, and protein were recovered at the start after electrophoresis in Tris buffer pH 7.4, $\mu = 0.02$, and veronal buffer pH 8.6, $\mu = 0.01$. Topz ballotini glass beads (0.1 mm) were finally chosen as the supporting medium. It was first necessary to wash the glass beads with 1 l veronal buffer pH 8.6, 1 l physiological saline, 2 l 0.01 N sodium hydroxide and then with distilled water to pH 7.0, since it was found that the nucleoproteins would not migrate unless the glass beads were treated in this way. The buffer employed for electrophoresis was tris(hydroxymethyl)aminomethane (Tris) pH 7.4, $\mu = 0.02$. The glass bead-block was prepared as described previously by RHODES *et al.*[13].

20 mg of the nucleoprotein was dissolved in 0.1 ml water and dry glass beads were added to make a paste which was placed in a small transverse trough about 4 cm from the cathode end of the block. After laying a piece of dry paper under the lid, the latter was sealed down with a strip of plaster and the current was applied for 41 h at 300 V, and 1.5 mA, at +4°.

After electrophoresis the glass block was cut into 30 × 1 cm segments each of which was eluted with 5 ml water, the glass beads being centrifuged down at 700 × *g*. Protein, RNA, and DNA in the supernatants were estimated by the Folin[7,7a], orcinol[8], and diphenylamine[9] methods respectively.

RESULTS

Table I illustrates a typical chemical analysis of the crude BCG and human strain E9656 nucleoproteins, and of the "purified" nucleoproteins. The latter were prepared by the addition of an equal volume of saturated ammonium sulphate to a solution of the crude nucleoprotein, as described under MATERIALS AND METHODS.

The solution of the BCG nucleoprotein was turbid, probably due to the lipid

TABLE I

CHEMICAL ANALYSIS OF THE BCG AND HUMAN STRAIN E9656 NUCLEOPROTEINS AFTER LYOPHILIZATION

Substance	*E9656*		*BCG*	
	Crude (%)	*"Purified"* (%)	*Crude* (%)	*"Purified"* (%)
Protein (Biuret)	40.1	30.8	(> 100) (turbid lipid?)	71.3
DNA Dische	24.0	25.2	24.0	18.2
RNA	9.6	7.2	8.2	6.0
P	5.4	5.3	1.6	1.2
N	6.0	9.1	3.2	8.0

present, which may account for the excessively high protein value found with the biuret reagent.

The nucleoproteins showed an absorption maximum at 260 mμ and a minimum at 235 mμ. The curves are shown in Fig. 1 and 2. Both indicate that some of the nucleic acid has been lost in the "purification" procedure since otherwise the maximum at 265 mμ would be considerably higher.

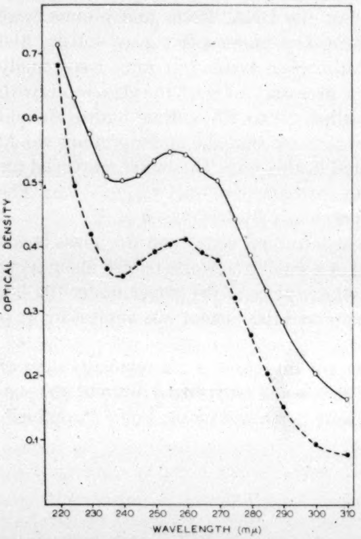

Fig. 1. u.v. spectrum of BCG nucleoproteins, 0.1 mg/ml in water: A = crude nucleoprotein; B = "purified" nucleoprotein.

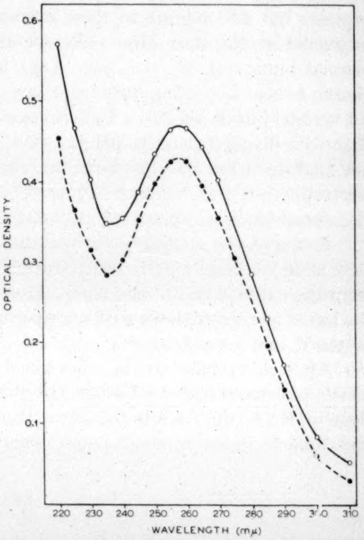

Fig. 2. u.v. spectrum of nucleoproteins from human strain E9656, 0.1 mg/ml in water: A = crude nucleoprotein; B = "purified" nucleoprotein.

Only one sedimentation peak is evident in the analytical ultracentrifuge (see Fig. 3 and 4), the crude nucleoprotein from the human virulent tubercle *bacillus* strain E9656 giving a value of $S_{20} = 7.4 \cdot 10^{-13}$ and the crude BCG nucleoprotein $S_{20} = 8.8 \cdot 10^{-13}$. The very sharp line shown by the BCG nucleoprotein may conceivably be due to the lipid present.

The results of zone electrophoresis of the BCG and E9656 nucleoproteins in glass beads are illustrated in Figs. 5 and 6. The curve for RNAP in both cases shows a major band and a second band of higher mobility in the same position as the DNAP. Protein was found across the whole block, but the main portion appears to be bound as RNA protein of slower mobility. The separation of the DNAP from the first RNAP is very clear-cut; the proportion of DNAP to RNAP in the second band being about 4 or 6:1, whereas there is about twice as much protein in the slower moving RNP band than in the faster moving RNAP zone. The absorption, in the diphenylamine test, which is apparent at the start in the electrophoresis pattern of the BCG nucleoprotein (Fig. 6),

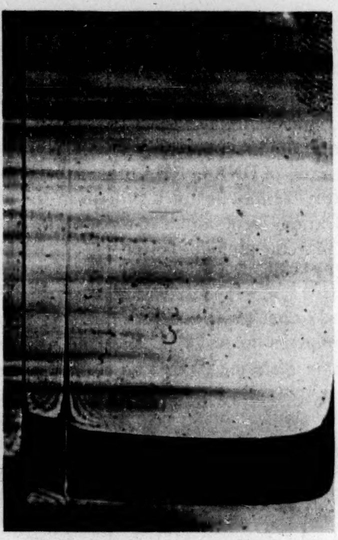

Fig. 3. Ultracentrifuge pattern of crude nucleoprotein from human virulent tubercle *bacilli*. 0.6% phosphate buffer, pH 7.3. Rotor speed 36,000 r.p. m. Model E Spinco analytical ultracentrifuge. Exposure 44 min after maximum speed. Angle 60°. $S_{20} = 7.4 \cdot 10^{-13}$.

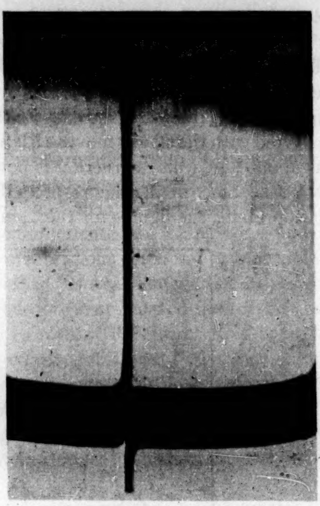

Fig. 4. Ultracentrifuge pattern of crude nucleoprotein from BCG 0.6% in phosphate buffer pH 7.3. Rotor speed 36,000 r.p.m. Model E Spinco analytical ultracentrifuge. 33 min after maximum speed. Angle 70°. $S_{20} = 8.8 \cdot 10^{-13}$.

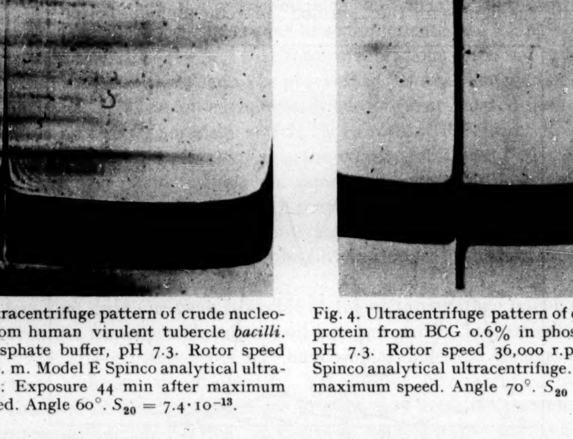

Fig. 5. Zone electrophoresis of crude nucleoprotein from human virulent tubercle *bacilli*. 20 mg in 0.1 ml Tris buffer, 0.01 μ, pH 7.3. Electrophoresis at 350 V, 1.5 mA, 41 h: △—△ RNA; ●—● DNA; ○—○ Protein.

is no doubt due to the lipid material present, since there is no typical diphenylamine colour in this region.

The "purified" nucleoproteins, which had been prepared by the addition of saturated ammonium sulphate, gave essentially the same type of result after electrophoresis as those illustrated in Figs. 5 and 6, namely, in both cases the DNAP again migrated with the faster moving RNAP, whilst the main portion of the protein was recovered with the slower moving RNAP. The ratio of DNA to RNA in the second zone was about 6:1, whereas there was now about 4–6 times as much protein in the first RNA zone. Since in all other respects the separations were similar to those of the crude nucleoproteins the figures for the "purified" proteins are not shown.

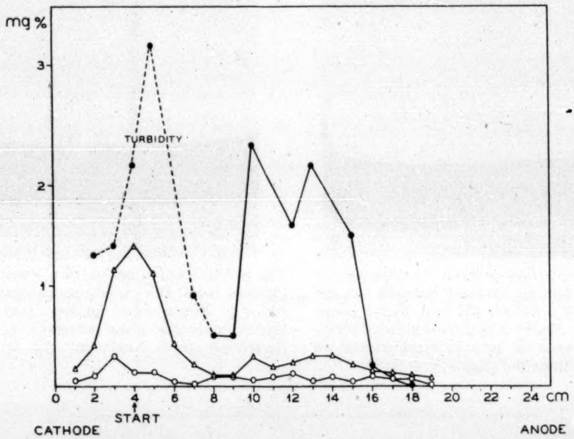

Fig. 6. Zone electrophoresis of crude nucleoprotein from BCG. 20 mg in 0.1 ml Tris buffer, 0.02 μ, pH 7.3. Electrophoresis at 350 V, 1.5 mA, 41 h: △—△ RNA; ●—● DNA; ○—○ Protein.

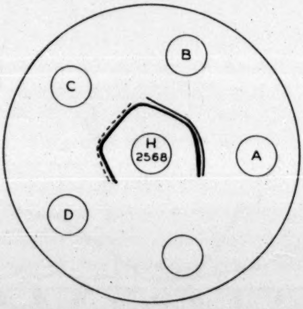

Fig. 7. Agar diffusion of nucleoproteins from BCG and human virulent strain E9656 in 1% agar. All nucleoproteins were dissolved in phosphate buffer pH 7.3 to give a solution containing 1 mg/ml: A = BCG crude nucleoprotein; B = BCG "purified" nucleoprotein; C = TB crude nucleoprotein; D = TB "purified" nucleoprotein; H2568 = horse antiserum to phenol killed E9656 strain human tubercle *bacillus*.

Immunologically the nucleoproteins are similar since they all cross-reacted when tested against horse antiserum to phenol killed human tubercle *bacilli* E9656 (see Fig. 7). The two fainter lines in C and D are probably similar to the second line in A and B but are not so clearly visible due to lack of that antigen in the nucleoprotein.

DISCUSSION

These results indicate that the nucleoprotein preparations from BCG and human tubercle *bacilli* E9656 show qualitatively the same type of result in zone electrophoresis in glass beads, in the ultracentrifuge, and in agar diffusion. The mycobacterial nucleoproteins described here can be separated into two RNAP zones and one DNAP zone in glass beads. The fact that these preparations could not be separated in starch and that the glass beads had to be pre-washed before use suggests that the glass beads might be acting as an ion-exchanger. Nucleoproteins isolated from *E. coli* have been separated by electrophoresis in starch, by PARDEE *et al.*[14], into 2 RNP zones and one DNAP zone in much the same way as in the experiments illustrated in Figs. 5 and 6. The DNAP was also found to have the same mobility as the faster moving RNP fraction. These workers were dealing with particulate DNAP and RNAP as shown by their sedimentation data in the ultracentrifuge of 40 S, 29 S, and 5 S whereas the preparations described here are in all probability no longer in the particulate form due to the method of preparation.

The fact that only one peak is visible in the ultracentrifuge might substantiate the view that the nucleoproteins described here are not in the particulate form. On the other hand, all the components may have similar molecular weights which would cause them to sediment simultaneously. Yet another interpretation of the results could be that the ribonucleoproteins are in some way attached to higher molecular weight components, such as DNA. It has been demonstrated that purified RNAP isolated from various sources does contain two sedimenting components[15–17] having molecular weights of $1.7 \cdot 10^6$ and $0.7 \cdot 10^6$. However, although there is evidence for two RNAP components after zone electrophoresis of the nucleoproteins described here no corroborative results were found in the ultracentrifuge.

It is perhaps not so surprising that the nucleoproteins from BCG and human virulent tubercle strain (E9656) cross react in agar diffusion against a horse antiserum to phenol killed E9656 tubercle *bacilli*. JENSEN AND LIND[18] and LIND AND TOLDERLUND[19] have shown that there is no difference in the specificity between PPD's from human and bovine type *bacilli* and BCG tested in human beings and guinea pigs. Other authors have also published similar findings[20–23].

Two types of antibodies to the nucleoproteins can be shown by the OUCHTERLONEY[12] technique. It is not known whether the proteins attached to the RNA or DNA give rise to these antibodies or whether they are directed against the RNAP and DNAP complex itself.

REFERENCES

[1] M. SPIEGEL-ADOLF AND F. SEIBERT, *J. Biol. Chem.*, 106 (1934) 373.

[2] F. SEIBERT AND D. WATSON, *J. Biol. Chem.*, 140 (1941) 55.

[3] F. SEIBERT, C. CRUMB AND M. SEIBERT, *J. Am. Chem. Soc.*, 72 (1950) 2678.

[4] K. PETRIK, *J. Bacteriol.*, 51 (1951) 539.

[5] E. CHARGAFF AND H. SAIDEL, *J. Biol. Chem.*, 177 (1949) 417.

[6] Tsumita and E. Chargaff, *Biochim. Biophys. Acta*, 29 (1957) 568.
[7] E. A. Kabat and M. M. Mayer, *Experimental Immunochemistry*, Charles C. Thomas, Illinois. U.S.A. 1948, p. 322.
[7a] W. Q. Wolfson, C. Cohn, E. Calvary and F. Ichiba, *Amer. J. Clin. Path.* 18 (1948) 727.
[8] W. Mejbaum, *Z. physiol. Chem. Hoppe-Seyler's*, 258 (1939) 117.
[9] Z. Dische, *Mikrochemie*, 8 (1930) 4.
[10] R. J. L. Allen, *Biochem. J.*, 34 (1940) 858.
[11] E. A. Kabat and M. M. Mayer, *Experimental Immunochemistry*, Charles C Thomas, Illinois, U.S.A. 1948, p. 282.
[12] O. Ouchterloney, *Arkiv Kemi*, 14 (1949) 1.
[13] J. M. Rhodes, E. Sorkin and S. V. Boyden, *Biochim. Biophys. Acta*, 25 (1957) 405.
[14] A. B. Pardee, K. Pargen and L. S. Prestidge, *Biochim. Biophys. Acta*, 23 (1957) 162.
[15] Yao-Cheng Ping, *Biochim. Biophys. Acta*, 37 (1960) 238.
[16] A. Z. Gieser, *Naturforsch.*, 136 (1958) 788.
[17] R. A. Brown, M. C. Davies and J. S. Colter, *Abstr. Biophys. Soc.* (*U.S.A.*) 1958 *meeting*.
[18] K. A. Jensen and P. Lind, *Acta Tuberc. Scand.*, 17 (1943) 37.
[19] P. Lind and K. Tolderlund, *Acta Tuberc. Scand.*, 17 (1943) 252.
[20] H. H. Green, *Vet. J.*, 102 (1946) 267.
[21] M. Magnusson, *Amer. Rev. Resp. Dis.*, 82 (1960).
[22] R. Parlett and G. R. Youmans, *Am. Rev. Tuberc. Pulmon. Diseases*, 73 (1953) 637.
[23] A. Lind, *Intern. Arch. Allergy and Appl. Immunol.*, 14 (1959) 264.

THE ROLE OF PHOSPHOPYRIDINENUCLEOTIDES IN THE METABOLISM OF CORTISOL BY PERIPHERAL TISSUE

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SUMMARY

1. Cortisol incubated with muscle tissue is metabolized to cortisone, dihydrocortisol, corticosterone, 11-β-hydroxyandrostenedione, Reichstein's substances E and U, dihydrocortisone, Kendall's Compound A and adrenosterone.

2. The reaction, cortisol → cortisone is TPN specific. DPN appears to inhibit the reaction. No evidence was obtained for the presence of a transhydrogenase in muscle tissue. Reduction at C-4, 5, and C-20 appears to be TPNH specific.

3. Studies of the metabolism of cortisone in muscle and liver indicate that the major product in liver is cortisol. The major product in muscle is Reichstein's substance U.

INTRODUCTION

Since the original observations that DPN plays a role in the metabolism of testosterone by the liver[1-3], the role of phosphopyridine-nucleotides has been studied extensively in the synthesis[4-7], hepatic degradation[5,6,8] and placental metabolism[8-12] of the steroid hormones. The advent of radioactive isotope techniques now makes possible studies of the relationship of these co-factors to the metabolism of steroid hormones in peripheral tissue, which is probably a site of action for several of the steroid hormones. In this study, it has been found that cortisol is metabolized to five products in muscle. One of the products (cortisone) is converted to four additional compounds. Several steroid conversions in muscle and connective tissue appear to be mediated through TPN and TPNH.

EXPERIMENTAL

1.0 g bovine* (psoas major) or mouse leg muscle (approx. 5% connective tissue, estimated histologically) or 0.2 g mouse facia (subdermal loose connective tissue) was incubated in a medium containing sodium phosphate buffer, 0.05 *M*, pH 7.35;

Abbreviations: DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.

* Obtained from slaughter house, kept on ice and teased free of fat and facia. Activity bore a relationship to the time taken for preparation. If too long an interim ensued the preparations were inactive. Age of the animal also seems to be a factor in the degree of activity.

$MgCl_2$, 0.004 M; KCl, 0.025 M; 16,000 counts/min cortisol (specific activity 4.04 μC/mg) or cortisone (specific activity 0.18 μC/mg and 13.9 μC/mg) at 37° for 2 h. Cofactors were employed in concentrations of $1 \cdot 10^{-3}$ and $1 \cdot 10^{-4}$ M. The soluble enzyme of muscle was prepared from the supernatant fraction (32,000 × g, for 1 h) of a homogenate (1 part tissue, 5 parts above buffer) by fractionating between 10 and 60 % saturated ammonium sulfate. A quantity of extract equivalent to 7.5 g of muscle tissue was employed for each experiment.

After incubation, three volumes of acetone were added to the incubation flasks (flasks contained 5–10 ml of incubation mixture) to precipitate the protein. The acetone was subsequently removed by vacuum distillation. The water residue was extracted with chloroform which was evaporated under reduced pressure. The chloroform residue was dissolved in chloroform–methanol (1:1) solution and chromatographed according to the methods of ZAFFARONI[13]. Radioactive areas of the chromatographs were located and analyzed by means of a strip feed counter[14,15]. Specific identification procedures are referred to below. In the oxidation procedures[15], 0.5 mg of chromium trioxide was employed for cleavage of the side chain; 0.1 mg for oxidation of the hydroxyl groups. Dryness of the chromium trioxide was assured by preliminary washing of the crystals with the glacial acetic acid. Control experiments with buffer indicated no steroid conversions.

RESULTS

Presumptive identifications of the radioactive products obtained in the experiments below were based on the following evidences:

Cortisol: Migrates with index cortisol in the chloroform system. Acetylation of combined index and radioactive areas gives compounds which migrate identically with cortisol acetate-21 (chloroform system). Mild oxidation of the acetate results in a compound identical with cortisone acetate-21. Oxidation of the unacetylated compound gives rise to adrenosterone.

Cortisone: Chromatographs with index cortisone in the chloroform system. Elution and acetylation of the combined forms of the steroids (radioactive and non-radioactive) results in derivatives which migrate in the chloroform and benzene systems at an identical rate to that of cortisone acetate. Oxidation of the unacetylated compound gives adrenosterone as a product.

Reichstein's substance E (4-pregnene-11-beta, 17-alpha, 20-beta, 21-tetrol-3-one): Chromatographs near the origin of the chloroform system. When the elution solvent (chloroform) is allowed to drop from the chromatogram (6 h) the radioactivity migrates with Reichstein's substance E. Acetylation of the compound forms a diacetate which migrates at the same rate as substance E-diacetate. Mild oxidation converts the radioactive compound to Kendall's compound E. Strong oxidation converts it to adrenosterone.

20-epi Reichstein's substance E: This compound is slightly more polar than substance E. It forms a diacetate which cannot be distinguished from substance E diacetate when chromatographed in the benzene system (6 h). Mild oxidation of the acetylated compound converts it to substance U acetate. This compound was observed primarily in liver after the incubation of cortisone. BERLINER *et al.*[16] have reported it to be a product of cortisol metabolism in connective tissue.

Reichstein's substance U (*4-pregnene-17-alpha, 20-beta, 21-triol-3,11-dione*): Migrates between Kendall's compounds E and F in the chloroform (front system.) When the eluent is dropped from the front of the chromatogram (4 h), the compound runs identically with the index compound. Upon acetylation, the compound forms a diacetate which positions itself with substance U-diacetate in the chloroform and benzene systems. Mild oxidation converts the free compound to compound E. Strong oxidation converts the free compound to adrenosterone.

Dihydro F (*pregnane-11-beta, 17-alpha, 21-triol-3, 20-dione*): This compound chromatographs slightly ahead of compound F in chloroform. When the solvent is dropped, the radioactive compound is resolved from the compound F and migrates identically with the index carrier compound. Acetylation of the radioactive compound forms a monoacetate which migrates identically with the acetate of index dihydrocortisol. Upon mild oxidation of the acetate a slightly less polar compound is formed which corresponds to dihydro compound E acetate.

Dihydro E (*pregnane-17-alpha, 21-diol-3, 11, 20-trione*): This compound chromatographs in a region slightly ahead of cortisone in the chloroform system. When the solvent is allowed to drop from the front of the chromatogram (3 h), the compound separates from cortisone. Acetylation gives rise to a monoacetate which migrates slightly ahead of cortisone acetate. An index compound was not available for this substance, but the chromatographic characteristics in respect to Kendall's compound E afford strong evidence that it is pregnane-17 alpha, 21-diol-3, 11, 20-trione.

Kendall's compound B (*4-pregnene-11-beta, 21-diol-3, 20-dione*): In the chloroform and benzene systems, this compound migrates identically with the standard compound. It forms a monoacetate which migrates with compound B acetate. Mild oxidation converts the compound (or its acetate) to Kendall's Compound A (or its respective acetate). Cleavage of the side chain by strong oxidation gives a highly polar compound corresponding to cholanic acid.

Kendall's compound A (*4-pregnene-21-ol-3, 11, 20-trione*): Chromatography in the benzene and chloroform systems demonstrated this compound to migrate identically with an authentic sample of Kendall's Compound A. A monoacetate is formed upon acetylation and runs at the same rate as the index acetate. Strong oxidation results in a highly polar compound corresponding to cholanic acid.

11-beta-hydroxyandrostene-3, 17-dione: This compound migrates with the nonradioactive carrier compound in the benzene and hexane-benzene systems. Mild oxidation converts it to adrenosterone. It does not form an acetate.

Adrenosterone (*4-androstene-3, 11, 17-trione*): This compound migrates with the carrier compound in the benzene and hexane–benzene systems. It does not form an acetate. Under the conditions of oxidation described above, this compound is not oxidized to another product.

Fig. 1 is a chromatograph of the steroid products of radioactive cortisol incubated with muscle. A similar spectrum of products has been observed with cultured human[17] and mouse fibroblasts[16]. In the presence of TPN, the respective peaks are elevated, while in the presence of DPN there appears to be an inhibition. A similar effect of TPN has been shown when cortisol is incubated with connective tissue (Fig. 2), albeit the oxidized cofactor does not seem to lend to an increase in the quantity of the high polar compounds in this latter tissue. When a TPNH generating system was incorporated with the connective tissue preparations, the high polar components were increased in quantity. The magnitude of cortisol metabolism in connective tissue is 3 to 4 times that of the muscle tissue.

To determine whether the specificity of the oxidation of cortisol to cortisone in respect to TPN and DPN was real or due to a membrane barrier, an active soluble extract was prepared and studied. In Fig 3, tracing No. 6 was obtained when no cofactors were added at a pH of 7.4. When TPN was added at this pH, a significant increase in the quantity of cortisone was formed (No. 2). At a pH of 9.2 in the presence of TPN, a large increase was observed. When the pH was lowered, as depicted in tracing No. 3, the quantity of cortisone formed was decreased. When the DPN was added at pH 7.4 and 9.2 (No. 8, No. 7), a small quantity of cortisone was observed; at pH of 6.2 (No. 9), only a trace was observed to be formed. In all experiments in which DPN was added, no increase of the quantity of cortisone was noted over the control. To determine whether a transhydrogenase possibly functioned in this system, DPN and TPN or TPNH in combination were incubated with the extract and radio-

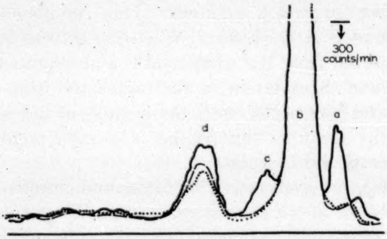

Fig. 1.

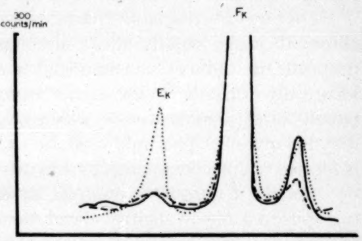

Fig. 2.

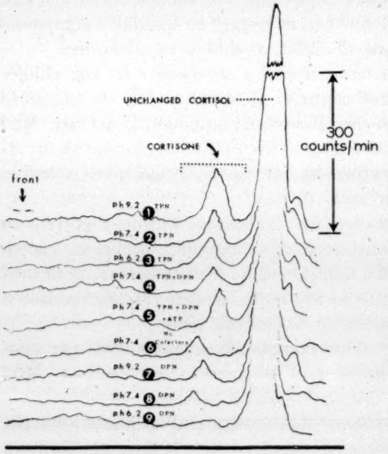

Fig. 3.

Fig. 1. Influence of TPN and DPN on cortisol metabolism by muscle tissue. The tracings are from radiochromatographic recordings obtained from cortisol products initially chromatographed in ZAFFARONI's chloroform-formamide system. The respective peaks were further chromatographed to give evidence of the following compounds: Peak a is largely composed of Reichstein's substance E, peak b is unchanged cortisol, peak c is largely dihydrocortisol and an as yet unidentified compound, and peak d is cortisone. The elevated area between cortisone and the front of the chromatogram contains Kendall's compound B and 11-beta-hydroxyandrostenedione. ——, TPN; ·········, DPN; – – –, control.

Fig. 2. Influence of TPN and DPN on cortisol metabolism in connective tissue. The respective radioactive areas after further chromatography resolve to essentially the same products as those referred to in Fig. 1. Subscript "K" refers to Kendall's steroid designation. ·········, TPN; – – –, DPN; ——, control.

Fig. 3. Influence of TPN and DPN on cortisol metabolism in a soluble extract of muscle tissue incubated at various pH's.

active cortisol. No evidence was found that DPN played an auxiliary role in these reactions (compare No. 4 with No. 6). Spectrophotometric studies also failed to demonstrate an effect of cortisol in the rate of reduction of either TPN or DPN. A suggestion that ATP might play a role in the reaction is seen in the tracing No. 5 in which ATP was added to the preparation together with TPN and DPN. Activity at the C-20 position is greatly diminished in the soluble preparation.

The data depicted in Fig. 3 is evidence that the oxidation of cortisol to cortisone is TPN specific. TPNH appears to be the specific cofactor in the reduction of cortisol at the 4–5 double bond and the C-20 ketone group. Cortisone seems to be the major steroid product of cortisol metabolism in unfortified muscle mince. The addition of TPN induces a small, but significant increase in the cortisone formed. A greater magnitude of increase is noted in the dihydro and C-20 reduced products when exogenous TPN is added. These observations are difficult to evaluate, but suggest a preferential affinity of TPN with the 11-dehydrocortisolase system in the endogenous preparation. The limited effect of exogenously added cofactor on the quantity of cortisone formed may be due to either a steric hindrance at the C-11 position or a limited quantity of the dehydrogenase.

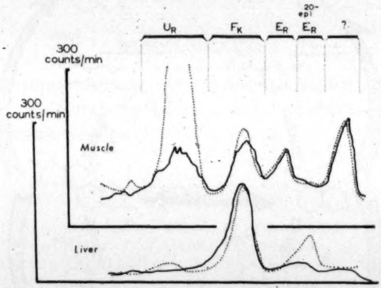

Fig. 4. A comparison of the metabolism of cortisone is muscle and liver. Glucuronic acid conjugation occurs with some steroid products in liver, but has not been detected in muscle tissue. Subscript "R" refers to Reichstein's steroid designation. ———, control; · · · · · ·, added TPN.

Studies of the reverse reaction (cortisone → cortisol) in both muscle and liver were also carried out in this investigation. Distinct differences were noted between the two tissues. When [4-^{14}C]cortisone is incubated with mouse muscle tissue, the dominating product is Reichstein's substance U; when incubated with mouse liver tissue the major product is cortisol (Fig. 4). The quantities of cortisol recovered from incubations of cortisone in muscle and liver tissue fortified with either TPN or TPNH do not appear to be significantly different from the controls (Fig. 4). However, the overall production of cortisol in liver seems to be increased with addition of coenzyme as Reichstein's substance E (20-epi), a secondary product of cortisol, is markedly increased. Fortification of the incubation mixtures with coenzyme appreciably increases the quantity of Reichstein's substance U in both tissues. The relative quantities of the products formed in muscle and liver are listed in Table I. Probable interrelations of cortisol, cortisone and their respective products is depicted in Fig. 5.

TABLE I

METABOLISM OF CORTISONE BY MUSCLE AND LIVER TISSUE

	Origin peak		E_R *or Epi* E_R		F_K		U_R	
	%*	*counts/min***	%	*counts/min*	%	*counts/min*	%	*counts/min*
1. Muscle								
Control	20.1	1,382	19.8	1,359	23.3	1,596	36.8	2,524
Addition of TPN								
1-	10.9	1,053	8.2	798	16.4	1,582	64.5	6,239
2-	12.2	937	10.2	774	14.8	1,131	62.8	4,820
2. Liver								
Control	5.6	1,392	13.4	3,308	69.0	17,036	11.9	2,932
Addition of TPN								
1-	6.7	1,949	28.4	8,197	52.0	15,031	12.8	3,702
2-	8.7	2,449	34.1	9,557	44.1	12,368	13.1	3,674

* Relative percentage of lipid soluble products.

** Counts/min were calculated from the area under the curve of the chromatograph as compared to a standard of known radioactivity.

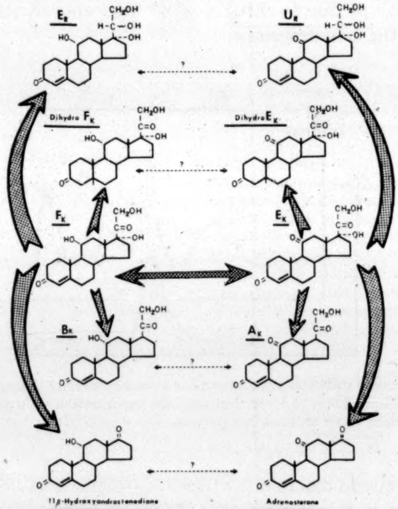

Fig. 5. Interrelations of cortisol and its products in muscle tissue.

DISCUSSION

It is apparent from the above studies that cortisol can be degraded in muscle tissue via at least five separate pathways, namely, cleavage of the C-17 side chain, oxidation at C-11, deoxygenation at C-17 and reduction at C-4, 5, and 20. If the mechanism of action of cortisol is via a chemical process (the possibility exists that cortisol may exert its influence by a physical means without involving a molecular change, *e.g.*, orientation at an interface), it is possible that one or more of these chemical reactions is involved in a key hormonal activation process. As cortisol is known to modify a number of physiological processes (electrolytic, glycogenic and anti-inflammatory activity, muscle work, etc.), it is also possible that the different chemical conversions are associated with separate physiological processes. That muscle tissue is a target organ of the cortico-steroids is evident from the work of INGLE[18] and SOLOMON AND SAYERS[19]. The relationship between the apparent endogenous affinity of TPN for 11-cortisoldehydrogenase and the mechanism of action of cortisol, as observed in the present experiments, remains to be determined.

BUSH[20] has suggested that the organism tends to maintain the 11-oxygen group of the corticosteroids in the reduced (hydroxyl) form. When 11-keto compounds were administered, a predominance of the 11-hydroxyl products were isolated in the urine. However, FUKUSHIMA *et al.*[21] have recently reported a predominance of the 11-keto urinary derivatives after administration of [4-^{14}C]cortisol. The present studies suggest that reduction of cortisone to cortisol is primarily a hepatic function. In muscle there does not seem to be a marked tendency for C-11 reduction in comparison to the reactions associated with the C-4,5, and 20 positions (Fig. 5). These observations are consistent with the recognition that cortisone administered systematically has comparable effects with cortisol, but when employed locally is much less active[22,23]. In respect to local anti-inflammatory reactions, cortisol has been estimated to be 7 to 8 times as effective as cortisone[24].

ACKNOWLEDGEMENT

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REFERENCES

[1] M. L. SWEAT AND L. T. SAMUELS, *J. Biol. Chem.*, 173 (1948) 433.

[2] M. L. SWEAT AND L. T. SAMUELS, *J. Biol. Chem.*, 175 (1948) 1.

[3] M. L. SWEAT, L. T. SAMUELS AND R. LUMRY, *J. Biol. Chem.*, 185 (1950) 75.

[4] M. L. SWEAT AND M. D. LIPSCOMB, *J. Am. Chem. Soc.*, 77 (1955) 5185.

[5] G. M. TOMPKINS, *Ann. N. Y. Acad. Sci.*, 82 (1959) 836.

[6] P. TALALAY, *Physiol. Revs.*, 37 (1957) 362.

[7] G. K. GRANT, *Ann. Repts. on Progr. Chem. (Chem. Soc. London)*, 52 (1955) 316.

[8] C. D. KOCHAKIAN, *IV Internat. Congr. Biochem.*, Symposia IV, Pergamon, N.Y., 1959, p. 196.

[9] E. D. HAGERMAN AND C. A. VILLEE, *J. Biol. Chem.*, 234 (1959) 2031.

[10] P. TALALAY AND H. G. WILLIAMS-ASHMAN, *Proc. Natl. Acad. Sci., U.S.*, 44 (1958) 15.

[11] V. P. HOLLANDER, N. HOLLANDER AND J. D. BROWN, *J. Biol. Chem.*, 234 (1959) 1678.

[12] L. J. LANGER AND L. L. ENGEL, *J. Biol. Chem.*, 233 (1958) 583.

[13] A. ZAFFARONI, *Recent Progr. in Hormone Research*, 8 (1958) 51.

[14] D. L. BERLINER, O. V. DOMINGUEZ AND G. WESTENSKOW, *Anal. Chem.*, 29 (1957) 1797.

[15] D. L. BERLINER AND H. A. SALHANICK, *Anal. Chem.*, 28 (1956) 1608.

[16] D. L. BERLINER AND T. F. DOUGHERTY, *Soc. Exptl. Biol. Med.*, 98 (1958) 3.

[17] M. L. SWEAT, B. I. GROSSER, D. L. BERLINER, H. E. SWIM, C. J. NABORS, JR. AND T. F. DOUGHERTY, *Biochim. Biophys. Acta*, 28 (1958) 595.

[18] D. V. INGLE, *J. Clin. Endocrinol.*, 10 (1950) 1312.

[19] N. SOLOMON AND G. SAYERS, *Federation Proc.*, 18 (1959) 446.

[20] I. E. BUSH AND V. B. MAHESH, *Biochem. J.*, 69 (1958) P. 9.

[21] D. K. FUKUSHIMA, H. L. BRADLOW, L. HELLMAN, B. ZUMOFF AND T. F. GALLAGHER, *J. Biol. Chem.*, 235 (1960) 2246.

[22] M. ZIFF, E. SCULL, D. FORD, C. MCEWEN AND J. J. BUNIUM, *A. M. A. Arch. Internal. Med.*, 90 (1952) 774.

[23] J. L. HOLLANDER, E. M. BROWN, JR., R. A. JESSAR AND C. Y. BROWN, *J. Am. Med. Assoc.*, 147 (1951) 1629.

[24] T. F. DOUGHERTY, in *Adrenal Cortex., Trans. of 2nd Conf.*, Josiah Macy, Jr. Fn., N.Y., 1950, p. 88.

THE METABOLISM OF 5-RIBOSYLURACIL

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SUMMARY

A dialyzed homogenate has been prepared from a pseudomonad that has been isolated from creek-mud and shown to contain enzymes which catalyze the following reactions:

5-ribosyluracil ⟶ uridine ⟶ uracil

Treatment of the cell-free extract with ammonium sulfate yields a fraction between 40 % to 60 % saturation in which only the enzymic transformation of 5-ribosyluracil ⟶ uridine is obtained.

The enzymic reaction requires phosphate and has a broad band type of optimum at pH 6.8 ± 0.5. In contrast to known phosphorylase reactions the activity is inhibited by arsenate. The data so far obtained do not conform to that expected for either of the two pathways known for the degradation of nucleosides. A mechanism of the type catalyzed by a mutase is postulated. In this mechanism the intermediary substance as cofactor is postulated to be 3,5-diribosyluracil, an as yet unknown substance. Certain analogues such as 5-methyluracil and 5-hydroxymethyluracil strongly inhibit the enzymic reaction while others such as 5-bromouracil, 5-aminouracil, and 5-hydroxydeoxyuridine are inert. 4-methyluracil is also inert. The influence which the carbon to carbon substituted analogues have on the enzymic reaction is strong evidence in favor of the allocation of ribose to position 5 in 5-ribosyluracil, an allocation which has been done on the basis of analogy rather than synthesis.

INTRODUCTION

A new component has been found as a minor constituent of certain ribonucleic acids[1]. This new component has been shown to be an isomer of uridine[2–4] in which the ribose is attached to position 5 of uracil rather than to position 3 and as such is termed 5-ribosyluracil. The ribosidic linkage in this case is unusual in naturally occurring nucleosides in that it is between two carbon atoms rather than between carbon and nitrogen. Such an unusual linkage opens the possibility that the metabolic pathway for the degradation of this nucleoside would be different from that of other known nucleosides. Certain aspects of the following study confirm such expectations.

Abbreviation: Tris, tris(hydroxymethyl)amino methane.

EXPERIMENTAL METHODS

Isolation of the microorganism

Samples of the soil from Strawberry Creek on the campus of the University of California, Berkeley, Calif. (U.S.A.) were suspended in 0.8 % solution of NaCl in the proportion of one part of soil to 9 parts of saline solution. The suspension was permitted to stand and sediment for 1 h at room temperature after which 1 ml of the supernatant suspension was incubated at 36° in 9 ml of the following solution: K_2HPO_4, 0.15 %; KH_2PO_4, 0.05 %; $MgSO_4 \cdot 7\,H_2O$, 0.02 %; 5-ribosyluracil, 0.1 %.

After growth of the organism became visible, aliquots of the suspension were chromatographed on paper according to techniques to be described later. The techniques chosen were such that certain chemical alterations in the structure of 5-ribosyluracil, if present, would be detected. Only one sample of mud from the many which were tried yielded an organism which produced detectable substances from the metabolism of 5-ribosyluracil. Aliquots from this sample were transferred successively in the foregoing medium until the culture was finally plated out in this medium containing 2 % agar.

The purity of the strain of the microorganism was maintained by keeping the parent culture in 0.1 % solution of 5-ribosyluracil. Organisms for seeding the bulk cultures were obtained by inoculating 10 ml of the following medium with a loopful of the suspension of the parent culture.

Difco yeast extract, 5 g; difco peptone, 5 g; glucose, 10 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7\,H_2O$, 0.2 g; $FeSO_4 \cdot 7\,H_2O$, 0.01 g; $MnSO_4 \cdot 7\,H_2O$, 0.01 g; NaCl, 0.01 g; Distilled H_2O, 1 l.

After mixing all ingredients together, the solution was adjusted to pH 6.8 by the use of 0.1 *M* solution of K_2HPO_4. 36 h at 28° produced sufficient growth for the subsequent inoculation of 1 l of the foregoing media in a 2 l flask. By trial, it was found that the optimum time for harvesting the cells was after 36 h of incubation. At this time the cells were in the logarithmic phase of growth and the acidity of the medium had increased such that the pH decreased from 6.8 to 6.6. This was the period during which the cells displayed maximum enzymic activity in the metabolism of 5-ribosyluracil. If the cells were permitted to grow for longer periods of time such that the cells reached a stationary phase with further decrease in the pH of the medium, enzymic activity toward 5-ribosyluracil rapidly decreased.

Cells were harvested by centrifugation for 30 min at 12,000 × *g* in a Lourdes centrifuge, refrigerated at 4°. After centrifugation the cells were washed once with a solution which contained 0.5 % NaCl and 0.5 % KCl and recentrifuged. Approximately 1.5 g of cells (wet wt.) were harvested from each liter of culture.

Preparation of homogenate, supernatant solution, and enzymically active fractions from the supernatant solution

After washing, equal weights of the packed bacterial cells and washed alumina (Alcoa A-301, 325 mesh) were mixed together in a mortar previously chilled in crushed ice. The cells and alumina were ground together[5] vigorously until the thick dry paste became moist. After grinding was completed, 0.02 *M* phosphate buffer at pH 7.0, in the amount of 1 ml/200 mg wet wt. of cells, was gradually added to dilute the thick paste. The mixture was finally centrifuged twice to remove unruptured cells, cell walls, and alumina. The first centrifugation was conducted at 16,000 × g for 10 min and the second at 16,000 × g for 20 min.

Part of the supernatant solution was used directly in the measurement of the enzymic activity of the whole cell-free extract, while the remainder was separated into three fractions by the use of ammonium sulfate at 4 to 5°, (a) 40 % saturation, (b) 60 % saturation, and (c) 80 % saturation. The precipitates which were formed on the addition of the ammonium sulfate were removed by centrifugation at 4° and 18,000 × g for 10 min.

The precipitates from the ammonium sulfate fractionations were dissolved in 0.002 *M* Tris buffer at pH 7.4. An amount of precipitate as obtained from 500 to 600 mg wet wt. of cells was dissolved in 1 ml of buffer and placed in cellophane dialysis tubing which was then suspended in 5 l of Tris buffer at room temperatures for 0.5 h and finally placed with constant stirring into a refrigerator at 4°. Dialysis was started in buffers at room temperature in order to allow any nucleic acids or polynucleotides which might be present in the precipitate to be fragmented into dialyzable substances and thus reduce the u.v. absorption of the enzyme solution to a minimum. Dialysis for 24 h with several changes of buffer gave satisfactory solutions free from ammonium sulfate and u.v. absorbing substances. The solutions were stored at 4° until used. The greatest enzymic activity was found in the 40–60 % ammonium sulfate fraction.

Preparation of 5-ribosyluracil

There are two published procedures for the preparation of 5-ribosyluracil, that of DAVIS AND ALLEN[1], and that of YU AND ALLEN[2]. The latter procedure takes advantage of the fact that commercial uridine as purchased from the Schwarz Laboratories, Inc., contains about 0.6 % of 5-ribosyluracil as a contaminant. It is less time consuming and hence less expensive to isolate 5-ribosyluracil from the commercial uridine as in the method of YU AND ALLEN than to isolate directly from ribonucleic acids.

Paper chromatography

Chromatograms were developed by the ascending technique on Whatman No. 1 filter paper. In all instances the *n*-butanol–water solvent of MARKAM AND SMITH[6,7] proved effective for the separation of 5-ribosyluracil from other nucleosides. Whatman No. 1 filter paper contains large quantities of u.v. absorbing materials which can be removed by layering the sheets of filter paper in 2 *N* acetic acid in a large flat container and autoclaving for 1 h at 15 lb. Finally the paper is washed several times in 95 % ethyl alcohol and dried. Photographic records of the chromatograms were made by the use of the technique of SMITH AND ALLEN[8], which is essentially that of exposing the chromatograms over Scona reflex paper to an 8 W u.v. lamp with peak emission at 2537 Å.

RESULTS

Enzymic activity of cell-free extracts

The results which were obtained in one experiment in which 500 μl of cell-free extract in phosphate buffer at pH 7.0 was incubated with 10 μM of 5-ribosyluracil at 31° are shown in Figs. 1 and 2. In order to obtain Fig. 1, aliquots of the incubation mixture were removed at 5, 10, 15, 30, 45, 60, 90 and 120 min and immediately brought to 75° to stop enzymic action. Each aliquot was then applied as a small compact area on Whatman No. 1 chromatography paper. After all aliquots were applied serially, the chromatograms were developed by submitting the paper to two separate ascending developments in butanol–water solvent. Each development required 18 h. Fig. 1 is a photograph of such a chromatogram taken under u.v. light. The areas in which the enzymic mixture was applied initially do not photograph since all u.v. absorbing material has moved out into three other areas. The substrate, 5-ribosyluracil, and the products of the reaction, uridine and uracil, have moved out in that order. Identification of the substances in the photographing areas was done by comparison of their R_F's in butanol–water and by elution from the paper and measurement of their absorption spectra in u.v. light.

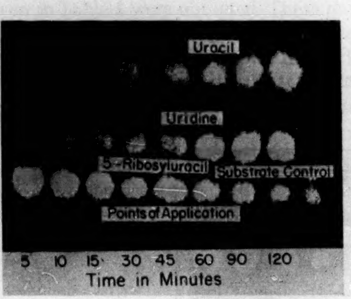

Fig. 1. Chromatogram in butanol–H_2O solvent of a rate study of the enzymic conversion of 5-ribosyluracil ⟶ uridine ⟶ uracil in a whole cell homogenate in phosphate buffer at pH 7.0. Ordinate, time in minutes. Labels which identify the u.v.-absorbing regions are placed over the areas.

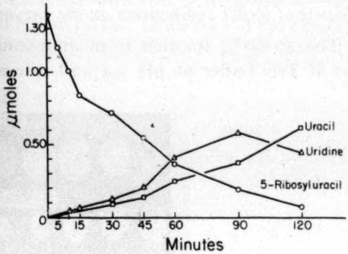

Fig. 2. Time course of the enzymic transformation of 5-ribosyluracil ⟶ uridine ⟶ uracil.

Fig. 2 was prepared from the data which were obtained by elution of the various areas on the chromatogram and measurement of the quantitative amounts of each substance at 2600 Å.

The disappearance of 5-ribosyluracil during the course of the reaction and the appearance and accumulation of uridine and uracil as shown in Figs. 1 and 2 indicate that the enzymic reactions occur in the following steps:

5-ribosyluracil ⟶ uridine ⟶ uracil

The first step in the reaction, 5-ribosyluracil ⟶ uridine has never been reported. The second step, uridine ⟶ uracil is well known. Uracil accumulates since the cofactor[9] necessary for its metabolism has been removed by dialysis.

Enzymic activity of certain fractions obtained by the use of ammonium sulfate

500 μl of each of the three ammonium sulfate fractions in phosphate buffer were incubated with 10 μM of 5-ribosyluracil at 31°. Aliquots of the incubation mixtures were taken as given in the foregoing paragraphs to obtain spectrophotometric data which showed that most of the enzymic activity was present in the fraction obtained at 40 to 60 % saturation and this showed only the first step of the reaction, viz.,

5-ribosyluracil ⟶ uridine

None of the figures that were obtained in this study are reproduced since in the interest of economy of space those figures which are included for the study of the influence of pH serve to document the foregoing statements.

The influence of pH upon the enzymic conversion of 5-ribosyluracil to uridine

Fig. 3 is the photograph of a chromatogram and Fig. 4 is the graph of the data which were obtained from the chromatogram in an experiment in which 100 μl of the 40 to 60 % fraction in various phosphate buffers were incubated with 0.82 μM of 5-ribosyluracil for 1 h at 31°. The pH-activity curve which is obtained is characteristic with a broad band type of optimum at pH 6.8 ± 0.5. Inactivation below pH 6.0 or above 7.9 is rapid.

Influence of buffer components on the enzymic reaction

The 40–60 % fraction from ammonium sulfate fractionation was dissolved in 0.002 *M* Tris buffer at pH 7.4 and exhaustively dialyzed against the same buffer.

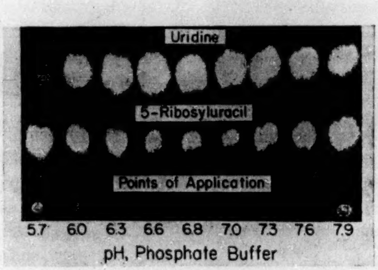

Fig. 3. Chromatogram in butanol–H_2O solvent of a study of the influence of pH on the enzymic conversion of 5-ribosyluracil ⟶ uridine. Ordinate, pH. Labels which identify the u.v.-absorbing regions are placed over the areas.

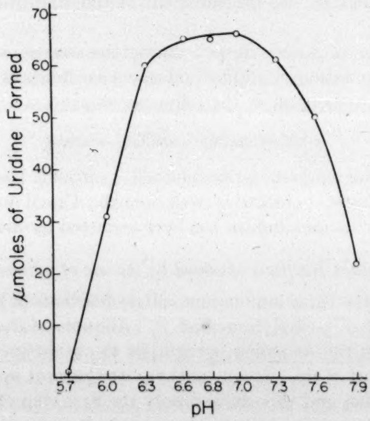

Fig. 4. pH Activity curve of the enzymic transformation of 5-ribosyluracil.

Aliquots of this enzyme-containing fraction were incubated for 2 h with 5-ribosyluracil which was dissolved in (a) Tris buffer at pH 7.4, (b) 10^{-3} *M* arsenate buffer at pH 7.4, (c) 10^{-3} *M* phosphate buffer at pH 7.4, and (d) a mixture of 10^{-3} *M* phosphate and 10^{-3} *M* arsenate buffers at pH 7.4. At the end of the incubation period samples of the mixture were withdrawn and chromatographed in butanol–water solvent. No conversion of 5-ribosyluracil ⟶ uridine could be demonstrated in either Tris buffer alone or arsenate buffer alone. When aliquots of the incubation in 10^{-3} *M* phosphate plus 10^{-3} *M* arsenate were withdrawn at 15, 30, 45, 60, 90 and 120 min intervals and chromatographed such that areas could be eluted and the amounts of 5-ribosyluracil remaining at any given time could be measured spectrophotometrically, the data given in Table I were obtained. These data show that the presence of arsenate ion in phosphate produces a lag in the reaction which results in an 80 % inhibition during the first 15 min of the reaction. After the initial lag no further inhibition in the reaction is observed.

TABLE I

COMPARISON OF ENZYMIC CONVERSION OF 5-RIBOSYLURACIL ⟶ URIDINE

A, phosphate buffer, pH 7.0; B, phosphate buffer + arsenate buffer, pH 7.0.

Time in minutes	*Control uridine μM A*	*Uridine μM B*	*% of lag*
15	0.067	0.014	79
30	0.115	0.039	66
45	0.155	0.093	41
60	0.177	0.127	28
90	0.214	0.155	27
120	0.242	0.168	30

Influence of certain 4- and 5-substituted pyrimidines on the enzymic reaction

The course of the reaction was studied in the presence of the following readily available analogues, (a) 4-methyluracil, (b) 5-bromouracil, (c) 5-hydroxymethyluracil, (d) 5-aminouridine, (e) 5-hydroxydeoxyuridine, and (f) 5-methyluracil (thymine). Prior to use in the enzymic system each of the substances was purified by chromatography in the butanol–water system. Each of the substances was mixed with 5-ribosyluracil and the active enzyme fraction in phosphate buffer at pH 7.0 and incubated for 2 h at 31°. At the end of the incubation period, aliquots were chromatographed, eluted and measured spectrophotometrically in the usual manner to obtain the data that are given in Table II. Strong inhibition of the enzymic reaction is displayed by those substances which contain a carbon to carbon linkage at position 5. Within the limits of the substances available, position 5 substitution which is not a carbon to carbon linkage such as –Br, –OH, or $-NH_2$ does not inhibit the reaction. Also substitution of a carbon to carbon linkage at position 4 as in 4-methyluracil does not inhibit the reaction. It should be noted at this point that the structure assigned to 5-ribosyluracil[2–4] is on the basis of analogy rather than synthesis. The inhibition which is observed to occur with 5-hydroxymethyluracil and 5-methyluracil is strong supporting evidence for the allocation of the ribose to position 5 in 5-ribosyluracil.

TABLE II

INFLUENCE OF CERTAIN PYRIMIDINES ON THE REACTION 5-RIBOSYLURACIL ⟶ URIDINE

	5-ribosyluracil μM	*Uridine recovered μM*	*% of inhibition*
4-methyluracil	0.450	0.442	—
5-bromouracil	0.450	0.440	—
5-hydroxymethyluracil	0.450	0.324	28
5-methyluracil	0.450	0.05	90
5-aminouridine	0.450	0.425	—
5-hydroxydeoxyuridine	0.450	0.439	—

Use of [2-^{14}C]uracil in an attempt to establish a mechanism for the enzymic reaction, 5-ribosyluracil ⟶ uridine

The foregoing data already point to the possibility that an unusual type of enzymic reaction has been found; however, it was felt necessary to conduct an experiment to decide whether or not the reaction were catalyzed by a nucleoside phosphorylase. If such were the case the transient formation of uracil could be expected to be followed by the transfer of the ribose to position 3. If radioactive uracil were to be added to the enzymatic reaction the incorporation of a small but definite percentage of the uracil into uridine could be expected.

[2-^{14}C]Uracil was purchased from Isotope Specialties Co., Burbank, California, and purified by chromatography in butanol–water solvent. After purification [2-^{14}C]-uracil in an amount containing $8 \cdot 10^5$ counts/min was mixed with 5-ribosyluracil and active enzyme fraction in phosphate buffer at pH 7.0 and incubated for 2 h at 31°. At the end of the incubation period the mixture was chromatographed in butanol–water solvent. In order to eliminate possible absorption of radioactivity the areas on the first chromatograms were eluted and rechromatographed in the same solvents. The area which contained the uridine was scanned and found to contain less than 150 counts/min. Further chromatographic purification gave no detectable radioactivity hence it can be inferred that free uracil is not an intermediate in the enzymic pathway.

DISCUSSION

At the present time the catabolism of pyrimidine nucleosides is known to occur by way of either one of two possible types of reaction, (a) an hydrolase type of reaction characterized by the addition of H_2O to the glycosidic linkage[10], or (b) a phosphorylase type of reaction characterized by the addition of phosphate to the glycosidic linkage[11].

If the enzymic hydrolysis of 5-ribosyluracil were to take place in an hydrolase type of reaction, uracil should be a direct product in the reaction. In the actual study of the reaction sequence in the breakdown of 5-ribosyluracil, uracil is formed only as a result of nucleoside phosphorylase reaction on uridine and not directly from the 5-irbosyluracil. The absolute requirement for phosphate ions also rules out known types of hydrolase reactions.

If the catabolism of 5-ribosyluracil were to follow the pathway of a phosphorylase type of reaction then both ribose-1-phosphate and uracil should be direct products of the reaction. Also arsenate could be substituted for phosphate with the expectation of the formation of ribose-1-arsenate. The enzymic transformation of 5-ribosyluracil

→ uridine is shown to have an absolute requirement for phosphate but to be inhibited by arsenate. One of the possible explanations of this apparent contradiction may be that the reaction, 5-ribosyluracil → uridine may take place in a minimum of two steps and that the ribose-1-phosphate which may be formed in the first step may be necessary for the second step. In the presence of arsenate, ribose-1-arsenate is formed but immediately undergoes spontaneous hydrolysis[12] to form ribose which is inactive. All attempts to separate the reaction into steps failed.

Since all of the experimental evidence fails to give unequivocal support that the enzymic action is either that of a hydrolase or a nucleoside phosphorylase an alternative may be that of a mutase type of reaction. Phosphoglucomutase is a well known mutase. The reaction catalyzed by phosphoglucomutase is given in the following equation with glucose-1,6-diphosphate as cofactor:

$$\text{Glucose-1-phosphate} + \text{glucose-1,6-diphosphate} \rightleftharpoons \text{glucose-1,6-diphosphate} + \text{glucose-6-phosphate}$$

In analogy with the foregoing reaction the transformation of 5-ribosyluracil to uridine can be written tentatively in the following manner with 3,5-diribosyluracil as cofactor:

$$\text{5-ribosyluracil} + \text{3,5-diribosyluracil} \rightarrow \text{3,5-diribosyluracil} + \text{3-ribosyluracil}$$

Since the enzymic reaction takes place by the use of exhaustively dialyzed extracts the presence of 3,5-diribosyluracil in the extracts can only be assumed in the event that it forms a non-dialyzable complex with the enzyme protein. The transformation as studied was irreversible.

If the reaction as studied should have the characteristics of a mutase as provisionally formulated then the reaction, 5-ribosyluracil → uridine could be classed also as an anabolic reaction accounting for the synthesis of both uridine and 3,5-diribosyluracil which are then later incorporated into ribonucleic acids. Uridine, of course, is a well known component of ribonucleic acids but 3,5-diribosyluracil has not yet been detected as a component of nucleic acids. The u.v. absorption characteristics of 3,5-diribosyluracil should be the same as those which are observed for uridine hence means other than spectrophotometric would have to be devised for its detection. The possible occurrence of 3,5-diribosyluracil as a component of ribonucleic acids becomes of paramount importance in an understanding of the structure inasmuch as this would provide the first unambiguous data on the possibility of branching in the ribonucleic acid structure.

ACKNOWLEDGEMENTS

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REFERENCES

[1] F. F. DAVIS AND F. W. ALLEN, *J. Biol. Chem.*, 227 (1957) 907.
[2] C. T. YU AND F. W. ALLEN, *Biochim. Biophys. Acta*, 32 (1959) 393.
[3] J. P. SCANNELL, A. M. CRESTFIELD AND F. W. ALLEN, *Biochim. Biophys. Acta*, 32 (1959) 406.
[4] W. E. COHN, *Biochim. Biophys. Acta*, 32 (1959) 569.
[5] H. MCILWAIN, *J. Gen. Microbiol.*, 2 (1948) 288.
[6] R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 45 (1949) 216.
[7] R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 45 (1949) 294.
[8] K. C. SMITH AND F. W. ALLEN, *J. Am. Chem. Soc.*, 75 (1953) 2131.
[9] E. S. CANELLAKIS, *J. Biol. Chem.*, 221 (1956) 315.
[10] C. E. CARTER, *J. Am. Chem. Soc.*, 73 (1951) 1508.
[11] L. M. PAEGE AND F. SCHLENK, *Arch. Biochem.*, 28 (1950) 348.
[12] L. A. MANSON AND J. O. LAMPEN, *J. Biol. Chem.*, 191 (1951) 95.

NON-ENZYMIC FORMATION OF DIAZOTIZABLE AMINE FROM RIBOSYLADENINE

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SUMMARY

Adenosine is cleaved non-enzymically in the presence of ketopentose, Cu^{++}, and pyrophosphate at 70° to yield small amounts of 5-amino-1-ribosyl imidazole. A similar diazotizable amine may be produced non-enzymically from ATP under conditions found in biological experiments. The preparation and characterization of the imidazole derived from adenosine are described. Two additional adenosine derivatives which are produced during the reaction have been partially characterized as 6-amino substituted compounds.

INTRODUCTION

ATP in the presence of pentose phosphate undergoes a non-enzymic degradation which leads to the formation of small amounts of diazotizable amine. This reaction may occur as an artefact in biological experimentation and illustrates some conditions which labilize the pyrimidine moiety of purine ring systems. The production of diazotizable amine from ATP is attributable to the ketopentose content of pentose phosphate preparations and requires the presence of cupric ion. While ketopentose phosphate appeared to be the most reactive carbohydrate component, other compounds of a ketose character, such as fructose and glycol aldehyde, promote formation

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Abbreviations: ATP, adenosine triphosphate; Tris tris(hydroxymethyl)aminomethane.

of the amine. Degradation of the purine to diazotizable amine at 37° was most extensive when ATP was employed; adenosine, adenosine monophosphate and diphosphate were less effective precursors of amine and these reactants required higher temperature and the presence of pyrophosphate. Neither adenine nor the phosphorylated ribosyl derivatives of other purines yielded diazotizable amine. The reaction most extensively studied, because of the availability of starting materials, has been that involving adenosine, fructose and cupric ion in pyrophosphate buffer. The diazotizable amine product has been tentatively identified, on the basis of its absorption spectra, chromatographic behavior, and color reactions, as 5-amino-1-ribosylimidazole. Two adenosine derivatives, both of which are tentatively considered to be 6-amino substituted compounds, are produced during the reaction and have been chromatographically separated and partially characterized.

EXPERIMENTAL

Materials

Adenosine triphosphate was obtained from Pabst and adenosine from Schwarz Laboratories, Inc. Ribose-5-phosphate was prepared from the barium salt (Nutritional Biochemical Corporation) by passing the acidified solution through a column of Dowex-50 H^+ resin. 5-phosphoribosylpyrophosphate was prepared enzymically, purified and assayed according to the methods described by KORNBERG *et al.*[1]. Ribose 5-phosphate isomerase, prepared from spinach and purified through the cysteine dialysis step, was employed for the synthesis of ribulose-5-phosphate and the latter compound was purified by column chromatography[2,3]. Prostatic phosphatase was prepared by dialyzing freshly homogenized tissue against distilled water for 16 h[4]. Ribulose and xylulose were prepared by epimerization of the aldose in pyridine[5].

Analytical methods

Ketopentose was determined by the cysteine–carbazole reaction[6], diazotizable amine by the method of BRATTON AND MARSHALL[7], imidazole by the KOESSLER-HANKE modification of the PAULY reaction[8], and ribose by the method of MEJBAUM[9]. Periodate titrations were performed according to DYER[10]. Sugars were identified, after dephosphorylation with prostatic enzyme when indicated, by chromatography in phenol–water (9:1) (see ref. 2).

Diazotizable amine was detected on paper by a modification of the method of SMITH[11] in which a saturated solution in absolute alcohol of N(1-naphthyl) ethylenediamine dihydrochloride was substituted for the Ekman reagent. Ribose was detected by periodate dip[12], reducing sugar by aniline phthalate[13] and ketose by a trichloracetic–orcinol spray[14]. Imidazole was detected by means of the PAULY reaction[15].

Reactions with ATP

At 37° in Tris–HCl buffer (0.5 *M*, pH 7.0), ATP and ribose-5-phosphate react in the absence of tissue extracts to yield a trace amount of amine which was detected by the BRATTON-MARSHALL reaction. When cupric ion was added to the mixture the yield of amine was increased 10-fold. The optimal concentration of cupric ion was a function of the concentration of ATP and pentose phosphate employed. In incubation mixtures of 1 ml containing 10 µmoles ATP and 16 µmoles pentose phosphate, optimal cupric ion was 2.5 μmoles. Purification of ATP through anion exchange chromatography[16] did not alter its reactivity for amine production in this system. Bromine oxidation of ribose phosphate, which degrades aldopentose compounds[3], did not decrease the yield of amine. Since commercial ribose-5-phosphate preparations contain ketopentose which survives the latter treatment, enzymically synthesized ribulose-5-phosphate was used in the reaction mixture. As shown in Table I, treatment of ribose-5-phosphate with spinach epimerase increased the content of carbazole reacting material (ketopentose) 3-fold and incubation of this mixture with ATP and Cu^{++} gave an approx. 4-fold increase of amine. Dephosphorylation of the pentose mixture with prostatic phosphatase prior to incubation with ATP and Cu^{++} resulted in 50 % lower yields of amine. When partially purified ribulose and xylulose, synthetically prepared by pyridine epimerization, were employed, the yield of amine was lower than that resulting from incubation with enzymically synthesized ketopentose of comparable concentration (based on carbazole reacting material). This may be due to trace contamination with pyridine in the synthetic product which interferes with the interaction of Cu^{++} and purine. 5-phosphoribosyl-1-pyrophosphate did not react to yield diazotizable amine, nor did glucose, glucose-1-phosphate, glucose-6-phosphate, ribose, xylose or arabinose. At 37° fructose gave a low yield of amine which was increased by incubating the reaction mixture at 80°. The relationship of ketopentose concentration to amine production was studied under the conditions described above. Amine production was optimal at 37°, pH 7 in Tris buffer (0.025 M) when the ratio of ATP, ketopentose-phosphate and Cu^{++} was 15:4:2.5. Amounts of ketopentose in excess of 0.004 M produced no further increase in amine production; high concentrations of sugars were inhibitory. The proportion of Cu^{++} ion necessary for optimal yield of amine was dependent upon the relative proportion and amount of the other two reactants and the buffer. In general, cupric ion concentrations in molar excess with respect to the carbohydrate strongly inhibited the reaction. The requirement for cupric ion could not be replaced by Cu^{+}, Mg^{++}, Mn^{++}, Fe^{++}, Fe^{+++}, Co^{+}, Zn^{++} or Ca^{++}. The reaction was completely inhibited by 10^{-3} M ethylenediaminetetraacetic acid. When mixtures were incubated anaerobically in the presence of N_2 the yield of amine was greatly decreased. An optimal yield of amine was obtained by shaking the mixture in air throughout the incubation period. However, the yield was not increased by an atmosphere of pure O_2.

TABLE I

EFFECT OF TREATING RIBOSE-5-PHOSPHATE WITH RIBOSE-5-PHOSPHATE ISOMERASE

Reaction mixture contained in 1 ml of 0.025 M Tris buffer, pH 7.0: ATP 4.7 μmoles, $CuCl_2$ 0.1 μmole and ribose-5-phosphate 44 μmoles. The mixture was incubated at 37° in an atmosphere of air for 3 h.

	Cysteine-carbazole reaction Optical density/ml	*Bratton Marshall reaction Optical density/ml*
Pentose-phosphate	700	115
Treated with isomerase	2,050	400
Then phosphatase	2,000	214

The amount of diazotizable amine in the reaction mixtures increased slowly during the first 3 h of incubation at 37°, then declined. During this period ketopentose concentration was continually decreasing whether all the reactants were present or not, and addition of ketopentose at 30 min intervals did not increase the yield. In 0.1 N acid or alkali the arylamine was rapidly destroyed at room temperature. It was adsorbed on Dowex-1-formate but could not be recovered with formic acid or buffered formate. On paper chromatography the amine moved in several solvent systems as a band which partially overlapped ATP.

The yield of BRATTON-MARSHALL reacting amine from ATP under optimal conditions was about 1 %, based on a molar extinction coefficient at 504 mμ of 24,000 for the colored product of the diazotized arylamine. This value is approximately that (24,600) reported for aminoimidazoleribosylphosphate at 500 mμ (see ref. 17) and for aminoimidazolecarboxamideribosylphosphate (26,400) at 540 mμ (see ref. 18). The absorption maximum of the compound formed in the BRATTON-MARSHALL reaction with the amine derived from ATP was 504 mμ.

Reaction with adenosine

Chromatographic evidence indicated that the arylamine derived from ATP was a ribosylpyrophosphate and that it was more labile than the ribosylarylamine which could be produced under analogous conditions from adenosine. The conditions for amine production from adenosine, fructose and cupric ion were similar to those found optimal for ATP and ketopentose except that pyrophosphate was required and it was necessary to increase the temperature of the reaction mixture to 70°. The optimal pH was 7.6 to 8.0. The following conditions were employed. To 100 ml of distilled water 5 mmoles of adenosine, 5 mmoles of $Na_4P_2O_4$ and 3 mmoles of $CuCl_2$ were added and the mixture was heated at 100° for 20 min. This solution was added slowly with stirring over a period of 30 min to a solution of 2 mmoles of fructose in 50 ml distilled water at 70°. During this time a flocculent precipitate formed and the mixture became a pale yellow color. Incubation was continued at 70° and the mixture was vigorously aerated while the volume was maintained at 150 ml by frequent addition of distilled water. In the absence of aeration the yield of amine was decreased over 70 %. Continued amine production ceased after approx. 1.5 h during which time the precipitate disappeared and the mixture became brown. The sample was then concentrated under reduced pressure to 50 ml and allowed to stand at 4° for 16 h. The heavy precipitate which formed consisted mainly of adenosine. It was removed by filtration. About 90 % of the amine in the original solution

TABLE II

R_F IN SOLVENT SYSTEMS

1, *n*-propanol–0.3 N NH_4OH (3:1), Whatman No. 3, descending. 2, *n*-propanol–water (3:1), Whatman No. 1, ascending. 3, *n*-propanol–1 N acetic acid (3:1), Whatman No. 1 ascending. 4, *n*-propanol–0.2 N NH_4OH (3:1), Whatman No. 1 ascending. 5, *n*-butanol–glacial acetic acid–water (2:2:1), Whatman No. 1 descending.

	1	*2*	*3*	*4*	*5*
Compound I (arylamine)	0.65	0.43	0.38	0.42	0.42
Compound II	0.51	0.29	0.35	0.29	0.41
Compound III	0.39	0.20	0.21	0.18	0.33

was recovered in the filtrate. 50 ml of water were added to the filtrate and this solution was passed through a column of Dowex-1-formate, 3.2 cm² × 16 cm. The effluent was tested for arylamine at frequent intervals and after the accumulation of about 40 ml (the retention volume of the column) when the test became positive, collection of the sample was begun. The remaining original volume was collected and the column washed with 0.005 *M* sodium pyrophosphate (about 40 ml) until arylamine concentration began to decrease. These effluent solutions, which contained essentially all the arylamine applied and fructose, but virtually no cupric ion or adenosine, were combined, diluted to 200 ml and adjusted to pH 4 with 1 *M* formic acid. This solution was then passed through a Dowex-50-Na^+ column (2.6 cm² × 7 cm) and the column washed with 20 ml of water. The arylamine which was adsorbed to the column was recovered in highest yield by eluting with 0.03 *M* NH_4OH and collecting the first 15 ml of effluent after the pH rose above 6.5. The lability of the amine did not permit gradient elution with salt or acid. If the column operations were conducted rapidly and the solutions chilled in ice, the overall recovery of arylamine through this step was about 80 %.

Further purification of arylamine was achieved by paper chromatography. The ammonium hydroxide eluate was concentrated by vacuum distillation to 3–4 ml and applied to a 24 × 18 cm sheet of Whatman No. 3 paper. Descending chromatograms employing *n*-propanol–0.3 *N* NH_4OH (3:1) were run for 16 h. After the papers were dried, the u.v. absorbing bands were located, the paper was cut in corresponding strips and the strips were eluted with a small volume of distilled water. Three principal components were separated in this system as shown in Table II (adenosine has an R_F of 0.72 in solvent 1).

Compound I (*diazotizable amine*)

In the BRATTON-MARSHALL reaction this compound yielded a salmon-pink color with maximum absorption at 520 mμ in 5 % trichloracetic acid or 4 % perchloric acid. The amine was not acetylated by treatment with acetic anhydride. In several solvent systems (Table II) the compound gave a single spot which weakly adsorbed u.v. light, gave a strong arylamine reaction, a permanent yellow color in the PAULY reaction, and a positive periodate and ninhydrin reaction. When an extinction coefficient of 24,000 for the arylamine reaction product was employed[17], and ribose determined by the orcinol reaction, a ratio of arylamine to ribose of 0.8 was found. The ratio of less than unity is attributed to the lability of the arylamine during the process of separation and recovery from paper. On standing at room temperature at neutral pH the amount of BRATTON-MARSHALL reacting material decreased and the solution became yellow over a period of a few hours. Treatment with 0.1 *N* HCl at 100° for 10 min destroyed the arylamine. The u.v. absorption spectrum of the freshly prepared compound showed no maximum above 230 mμ in acid, neutral or alkaline solution. Attempts to convert the arylamine to a ureido derivative, which could be isolated as the picrate, were unsuccessful, probably because of the small amounts of compound employed and its lability. Based on the BRATTON-MARSHALL reaction the amount of chromatographically purified arylamine derived from 1.8 g of adenosine was 10 mg or 0.5 %; on the basis of ribose determined in the orcinol reaction the yield was 0.63 %.

Compounds (II) and (III)

Chromatographically isolated compounds (II) and (III) reacted with periodate on paper and gave positive orcinol reactions for ribose. The yield of these compounds from adenosine expressed as μmoles ribose determined in the orcinol reaction was: Compound II, 2.7 %, and compound III, 1.3 %. Hydrolysis of aliquots of these compounds (II and III) in 0.1 *N* NaOH at 100° for 5.5 h degraded both compounds to one u.v. absorbing component; this was identified as adenine on the basis of spectra and migration with the same R_F as adenine in several solvent systems; namely, in *n*-butanol–glacial acetic acid–water (2:1:1), in isoamyl alcohol saturated with 5 % Na_2HPO_4 and in isopropanol–1.2 *N* NH_4OH (5:3:1). The amount of adenine as determined by u.v. spectrophotometry on the eluate of the compounds separated by paper chromatography was approximately equimolar with the amount of ribose as determined by the orcinol reaction on a corresponding aliquot. When the compounds (II and III) were hydrolyzed in 2 *N* acetic acid at 100° for 2 h and the hydrolysate was chromatographed in *n*-butanol–glacial acetic acid–water (2:1:1) the original compounds were no longer present. Each parent compound gave rise to two new components; a carbohydrate with the R_F of ribose and a single u.v. absorbing spot with an R_F of 0.50 derived from (II) and 0.53 derived from (III). Hydrolysis in 0.1 *N* or 1.0 *N* HCl for 10 to 60 min at 100° degraded the compounds (II and III) to several poorly resolved fluorescent and u.v. absorbing products, none of which gave positive ninhydrin, Pauly, or BRATTON-MARSHALL tests.

The u.v. absorption spectra of the compounds (II and III) are shown in Figs. 1 and 2. Both compounds show hyperchromic shifts in acid and bathochromic shifts

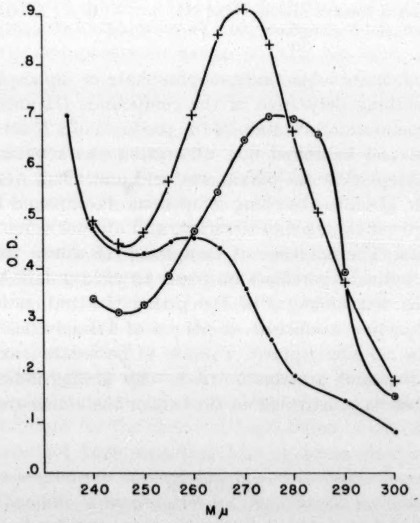


Fig. 1. The u.v. absorption spectra of compound II. +—+, in 0.1 *N* HCl; ●—●, in 0.05 *M* potassium phosphate, pH 7.4; ⊙—⊙, in 0.1 *N* NaOH.

in alkali. Dissociations were found at pH 3.7 and 12.0 for both compounds by plotting the spectral change at 270 mμ as a function of pH. The u.v. absorption spectra of the compounds (II and III) were not changed by treatment with nitrous acid under conditions which deaminate adenosinemonophosphate or diphosphopyridine nucleotide[19]. The u.v. absorbing derivative of the compounds (II and III) obtained by chromatographic separation and elution of the products of 2 *N* acetic acid hydrolysis contained no ribose and exhibited u.v. absorption characteristics similar to the parent compound except that the maxima in acid and alkali were shifted 5 mμ to a shorter wavelength. The u.v.-absorbing component of compound II did not consume periodate, as determined by arsenite titration, and did not react with periodate on paper chromatograms. The aglycone of compound III slowly consumed periodate in solution and reacted with periodate on paper to yield a dark blue spot after the benzidine spray. The stoichiometry of the periodate titration is not established. However, if the extinction coefficient at pH 7.0 of III-aglycone is assumed to be the same as that for adenine, approx. 1 μmole of periodate is consumed/μmole of purine. Since the compound contains no ribose this finding indicates that a group reacting with periodate is substituted on the ring or the amino group of the adenine moiety.

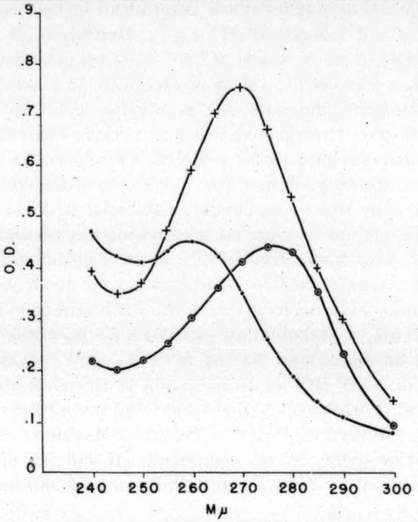


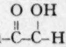
Fig. 2. The u.v. absorption spectra of compound III. +—+, in 0.1 *N* HCl; ●—●, in 0.05 *M* potassium phosphate, pH 7.4; ⊙—⊙, in 0.1 *N* NaOH.

DISCUSSION

The arylamine derived from adenosine in a reaction with fructose, Cu^{++} and pyrophosphate gives a Pauly test for imidazole, a BRATTON-MARSHALL reaction for non-acetylatable arylamine (maximum 520 mμ), exhibits no u.v. absorption maxima above 230 mμ, and contains approximately equimolar amounts of arylamine and ribose. This evidence supports the proposed structure of 5-amino-1-ribosylimidazole. The analogous reaction of ATP, ketopentose phosphate, and Cu^{++} is believed to produce the pyrophosphate derivative of 5-aminoimidazoleribosyl-5′-phosphate. However, this product has not been chromatographically purified and characterized and the proposed identity is supported only by analogy.

The two adenosine derivatives, which may be intermediates or by-products in the reaction leading to arylamine production from adenosine and fructose, have substituents which are cleaved in 0.1 N NaOH to yield adenine. These compounds show pronounced u.v. absorption shifts in acid and alkali and treatment with nitrous acid does not change their spectrophotometric characteristics. The findings are consistent with substitution on the 6-amino position of adenosine. The two derivatives apparently differ from each other with respect to the character of the substituent group. In the aglycone of compound III, the substituent reacts with periodate, while this is not the case with compound II. Both substituents may be low molecular weight fragments derived from fructose, but the nature of the groups is not known.

The reaction leading to ribosylaminoimidazole formation from adenosine and its derivatives is complex and probably involves equilibria which permit the accumulation of only small amounts of reactive intermediates. Copper may participate in several phases of the reaction. It is significant that adenine, which chelates strongly with cupric ion and simultaneously loses a proton[20], does not give rise to amine; a ribosyl substituent on N 9 of adenine is essential for the reaction. Since ATP does not require additional pyrophosphate to serve as an arylamine precursor, while adenosine does, it would appear that pyrophosphate enters into the formation of the cupric chelate. A formulation of the reaction of Mg^{++} with the 6-amino group, N-7, and the pyrophosphate group of ATP has been discussed by SZENT-GYÖRGY[21].

The ketose configuration$-\overset{\mathrm{O}}{\overset{\|}{\mathrm{C}}}-\overset{\mathrm{OH}}{\overset{|}{\mathrm{C}}}-\mathrm{H}_2$, which is essential to the carbohydrate component of the degradative reaction, may add to the chelated purine at N-1, the 6-amino, or at both positions. Previous studies have demonstrated that purines substituted in these positions exhibit characteristic lability. SHAW[22] found that benzyl substitution on N-1 of inosine rendered the purine labile to alkaline hydrolysis with the resultant formation of ribosylaminoimidazole benzylcarboxamide. In BROWN's laboratory[23] it was shown that adenine N-1 oxide cleaved in acid far more readily than adenine to yield aminoimidazolecarboxamide. N-succinoadenine, a compound substituted on the 6-amino group, was also found to be degraded in acid to aminoimidazolecarboxamide[24], a reaction which probably involves an attack of the substituent carboxyl group upon N-1 of the purine ring.

While ribosylaminoimidazole has been detected in the culture fluid or bacteria and molds[25–27], conditions for the chemical degradation of adenosine to the ribosylaminoimidazole have not been reported. The evidence cited above[22–24] indicates that the carboxamide group of aminoimidazolecarboxamide is resistant to cleavage in acid or alkali. Therefore, a mechanism for degradation for adenosine to aminoimidazole probably involves removal or complexing of both N-1 and the 6-amino group in such a manner as to yield the labile 5-amino-4-carboxyimidazole riboside which decarboxylates to the amino compound. The dependence of the overall reaction upon aerobic conditions implies an oxidative step in the sequence.

An understanding of the mechanism of enzyme reactions which open the pyrimidine portion of purine ring systems may be developed by further study of the chemical degradations described.

REFERENCES

[1] A. Kornberg, I. Lieberman and E. S. Sims, *J. Biol. Chem.*, 215 (1955) 393.
[2] M. Tabachnick, P. A. Srere, J. Cooper and E. Racker, *Arch. Biochem. Biophys.*, 74 (1958) 315.
[3] P. A. Srere, J. Cooper, M. Tabachnick and E. Racker, *Arch. Biochem. Biophys.*, 74 (1958) 295.
[4] Schmidt, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. II, Academic Press, Inc., New York, New York, 1955, p. 523.
[5] P. A. Levene and R. S. Tipson, *J. Biol. Chem.*, 115 (1936) 731.
[6] B. Axelrod, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. I, Academic Press, Inc., New York, New York, 1955, p. 363.).
[7] A. C. Bratton and E. K. Marshall, *J. Biol. Chem.*, 128 (1939) 537.
[8] K. K. Koessler and M. T. Hanke, *J. Biol. Chem.*, 39 (1919) 497.
[9] Z. Dische, in E. Chargaff and J. N. Davidson, *The Nucleic Acids*, Vol. I, Academic Press, Inc., New York, New York, 1955, p. 301.
[10] J. R. Dyer, in D. Glick, *Methods of Biochemical Analysis*, Vol. III, Interscience Publishers, Inc., New York, New York, 1956, p. 111.
[11] I. Smith, *Chromatographic Techniques*, William Heineman Medical Books, Ltd., London, 1958, p. 127.
[12] H. T. Gordon, W. Thornburg and L. N. Werum, *Anal. Chem*,. 28 (1956) 849.
[13] S. M. Partridge, *Nature*, 164 (1949) 443.
[14] R. Klevstrand and A. Nordal, *Acta. Chem. Scand.*, 4 (1950) 1320.
[15] B. A. Ames and H. K. Mitchell, *J. Am. Chem. Soc.*, 74 (1952) 252.
[16] R. B. Hurlbert, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. III, Academic Press, Inc., New York, New York, 1957, p. 785.
[17] B. Levenberg and J. M. Buchanan, *J. Biol. Chem.*, 224 (1957) 1005.
[18] J. G. Flaks, M. J. Erwin and J. M. Buchanan, *J. Biol. Chem.*, 228 (1957) 201.
[19] N. O. Kaplan, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. III, Academic Press, Inc., New York, New York, 1955, p. 874.
[20] T. R. Harkins and H. Freiser, *J. Am. Chem. Soc.*, 80 (1958) 1132.
[21] A. Szent-Györgyi, in O. H. Gaebler, *Enzymes: Units of Biological Structure and Function*, Academic Press, Inc., New York, New York, 1956, p. 393.
[22] E. Shaw, *J. Am. Chem. Soc.*, 80 (1958) 3899.
[23] M. A. Stevens, D. I. Magrath, H. W. Smith and G. B. Brown, *J. Am. Chem. Soc.*, 80 (1958) 2755.
[24] C. E. Carter, *J. Biol. Chem.*, 223 (1956) 139.
[25] S. H. Love and J. S. Gots, *J. Biol. Chem.*, 212 (1955) 647.
[26] D. P. Lones, C. Rainbow and J. D. Woodward, *J. Gen. Microbiol.*, 19 (1958) 146.
[27] H. Freidman and A. G. Moat, *Arch. Biochem. Biophys.*, 78 (1958) 146.

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INVOLVEMENT OF NEWLY-FORMED PROTEIN IN THE SYNTHESIS OF DEOXYRIBONUCLEIC ACID

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SUMMARY

By the combined use of thymine starvation and chloramphenicol, it was shown that DNA synthesis requires the formation of protein which may precede or accompany it.

INTRODUCTION

Escherichia coli 15 t^-h^- (thymine- and histidine-less strain) is unable to synthesize both thymine and histidine. As described by BARNER AND COHEN[1], this strain shows a characteristic death as a consequence of unbalanced growth during thymine starvation in a medium lacking thymine but otherwise complete. During thymine starvation there is little, if any, synthesis of DNA but a vigorous synthesis of RNA and protein takes place. If after thymine starvation, the bacteria are suspended in medium now provided with thymine but lacking histidine, there is a remarkable synthesis of DNA without any concomitant net increase of either RNA or protein. Experiments were therefore carried out to examine the role of prior protein synthesis in this synthesis of DNA.

METHODS

Cells of *E. coli* 15 t^-h^- grown on a glucose-salts medium (GRAY AND TATUM's medium[2] with 0.1 % glucose) supplemented with thymine (5 μg/ml) and L-histidine (10 μg/ml) were harvested during the exponential phase of growth, washed twice and suspended in two lots of thymine starvation medium (glucose-salts with histidine but no thymine) at a concentration of approx. $4 \cdot 10^8$/ml. Chloramphenicol (20 μg/ml) was added to one of these lots. Both were incubated at 37° with shaking and aliquots withdrawn at 0, 30, 60 and 120 min, washed twice, and resuspended in thymine medium (glucose-salts with thymine but no histidine). These timed aliquots from the two original lots were further divided into two identical series. To one of these chloramphenicol was added, and all cultures were incubated for an additional 120 min.

Throughout the experiment 20 ml aliquots of the bacterial suspensions were sampled from each series of flasks and treated with 5 % TCA in the cold. After sedimentation the pellets were washed in cold 5 % TCA and then extracted by 5 %

Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; TCA, trichloroacetic acid.

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TCA at 90° for 15 min. Aliquots of this extract were used for the estimation of both DNA and RNA by the diphenylamine method[3] and orcinol reaction[4], respectively. The indole reaction[5] was also used for DNA estimation in parallel with the diphenylamine method to confirm the measurements. Protein was estimated by the use of Folin reagent[6] taking aliquots from the TCA precipitated fraction.

All experiments reported in this paper were repeated at least once with similar results.

RESULTS AND DISCUSSION

E. coli 15 t^-h^- was found to synthesize a significant amount of DNA in the absence of exogenous histidine when exponentially growing cells were transferred to a thymine medium without previous starvation. Thymine starvation prior to incubation in thymine stimulated an even faster DNA synthesis. This is in good agreement with the report of BARNER AND COHEN[1] describing DNA synthesis in the absence of phenylalanine in *E. coli* 15 t^-pa^- (thymine- and phenylalanine-less).

During thymine starvation, while the synthesis of DNA was negligible, large amounts of RNA and protein were synthesized. As is clear from Table I, the longer the period of thymine starvation, the more DNA was synthesized during subsequent incubation in thymine medium. After 60 and 120 min of thymine starvation, the number of viable cells had already decreased to 36.4% and 0.4% respectively. Nonetheless, the cells were still metabolically active and there was an approximately three-fold increase in DNA after 2 h subsequent incubation in thymine medium. During this period, however, no significant net increase of either RNA or protein was observed and viable cell numbers remained constant. This is evidence for the separation of DNA synthesis from the synthesis of other macromolocules, although an active

TABLE I

EFFECT OF THYMINE STARVATION ON SUBSEQUENT SYNTHESIS OF DNA, RNA AND PROTEIN IN *E. coli* 15 t^-h^-

	Time of thymine starvation (min)	*Percent over initial value during thymine starvation*	*Percent over 0 min value during subsequent incubation in thymine medium*					
			No chloramphenicol			*With chloramphenicol*		
			0 min	*60 min*	*120 min*	*0 min*	*60 min*	*120 min*
Surviving cells per ml	0	100	100	90	107	100	90	105
	30	90.5	100	105	109	100	103	108
	60	36.4	100	91	100	100	96	93
	120	0.4	100	104	107	100	106	107
Amount of DNA per ml	0	100	100	150	151	100	163	180
	30	100	100	196	216	100	191	219
	60	102	100	224	289	100	220	286
	120	105	100	203	295	100	176	225
Amount of RNA per ml	0	100	100	127	133	100	190	209
	30	151	100	126	126	100	136	188
	60	228	100	103	102	100	150	147
	120	317	100	100	96	100	110	120
Amount of protein per ml	0	100	100	103	107	100	101	103
	30	120	100	100	99	100	100	98
	60	153	100	97	95	100	102	100
	120	222	100	95	93	100	100	100

turnover of some fractions of protein and/or RNA may not be denied. This critical point is now under investigation.

The mode of this DNA synthesis, occurring separately from an accompanying synthesis of protein, was investigated by the density gradient centrifugation method of MESELSON AND STAHL[7]. From an experiment reported elsewhere[8] evidence was obtained that DNA synthesis involves replication in non-dividing as well as in dividing cells. There may be a relation between this replication and the mutations claimed to be induced by thymine starvation[9–11].

No significant inhibitory effect of chloramphenicol on the synthesis of DNA was observed when chloramphenicol was added to the thymine medium after thymine starvation for different times as shown also in Table I, except for a little inhibition in the 120 min thymine starvation series. Chloramphenicol has been shown to inhibit protein synthesis in *E. coli* whereas nucleic acid synthesis continues in its presence[12]. The necessity for a concomitant net synthesis of protein during this period of active DNA synthesis is therefore ruled out. There is particular interest in the fact that the amount of protein synthesized during thymine starvation is proportional to the amount of DNA synthesized upon subsequent incubation with thymine. This relationship is much more obvious when chloramphenicol is added to the thymine starvation medium to prevent the synthesis of protein.

Although chloramphenicol failed to inhibit DNA synthesis in the thymine medium, it had a tremendous effect when added to the thymine starvation medium. The inhibition of the synthesis of protein by chloramphenicol during thymine starvation subsequently eliminated the stimulated synthesis of DNA which otherwise would have taken place in thymine medium. As shown in Table II, almost the same amounts of DNA were synthesized in each of the thymine media regardless of the length of previous thymine starvation. Chloramphenicol allowed RNA synthesis to a decreased extent in the thymine starvation medium, but this increase of RNA did not influence the amount of DNA synthesized afterwards. And also there appeared to be almost no breakdown of this RNA such as was previously observed by NEIDHARDT AND GROS[12] and HOROWITZ, LOMBARD AND CHARGAFF[13] in other amino acid auxotrophs of *E. coli* upon transferring the chloramphenicol treated cells to amino acid starvation medium without chloramphenicol. This is possibly due to the active turnover of RNA in thymine medium.

Another interesting finding is that chloramphenicol prevented thymineless death to a remarkable extent, *i.e.*, while in the control only 0.4 % of the cells were colony-formers after 2 h, 73.2 % remained so when chloramphenicol was present. BILLEN[14] reported a similar effect of chloramphenicol in preventing thymine-less death of *E. coli* 15 t⁻ (thymine-less).

The experiments reported here verify the hypothesis that prior protein synthesis is a prerequisite for subsequent DNA synthesis. They support similar findings of TOMIZAWA AND SUNAKAWA[15] on DNA synthesis in a system of multiplying bacteriophage, and of HAROLD AND ZIPORIN[16] and DRAKULIC AND ERRERA[17] on DNA synthesis of *E. coli* treated with ultra-violet light. Recently DOUDNEY[18] found a similar relationship between the synthesis of protein and DNA in a synchronous culture of *E. coli* by the use of chloramphenicol. From these results, it is suggested that the amount of protein previously synthesized may determine the amount of DNA formed subsequently. This protein seems to constitute, at least in part, a DNA-forming system.

The small amount of DNA formed in thymine medium, independent of the length of previous thymine starvation in chloramphenicol, is probably due to protein that was formed before the starvation.

TABLE II

EFFECT OF THYMINE STARVATION IN THE PRESENCE OF CHLORAMPHENICOL ON SUBSEQUENT SYNTHESIS OF DNA, RNA AND PROTEIN IN *E. coli* 15 t^-h^-

	Time of thymine starvation with chloramphenicol	*Percent over initial value during thymine starvation with chloramphenicol*	*Percent over 0 min value during subsequent incubation in thymine medium*					
			No chloramphenicol			*With chloramphenicol*		
			0 min	*60 min*	*120 min*	*0 min*	*60 min*	*120 min*
Surviving cells per ml	0	100	100	97	103	100	103	103
	30	100	100	—	100	100	—	100
	60	93.0	100	—	98	100	—	100
	120	73.2	100	—	100	100	—	102
Amount of DNA per ml	0	100	100	150	151	100	163	180
	30	102	100	129	145	100	131	144
	60	100	100	128	146	100	133	147
	120	104	100	121	145	100	122	134
Amount of RNA per ml	0	100	100	127	133	100	190	209
	30	154	100	93	105	100	114	123
	60	167	100	95	102	100	113	114
	120	180	100	99	103	100	111	110
Amount of protein per ml	0	100	100	103	107	100	101	103
	30	100	100	93	96	100	94	95
	60	100	100	90	105	100	96	100
	120	102	100	92	98	100	100	102

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REFERENCES

[1] H. D. BARNER AND S. S. COHEN, *J. Bacteriol.*, 74 (1957) 350.
[2] F. J. RYAN AND L. K. SCHNEIDER, *Genetics*, 34 (1949) 72.
[3] F. B. SEIBERT, *J. Biol. Chem.*, 133 (1940) 593.
[4] W. MEJBAUM, *Z. physiol. Chem., Hoppe Seyler's*, 258 (1939) 117.
[5] G. CERIOTTI, *J. Biol. Chem.*, 214 (1955) 59.
[6] O. H. LOWRY, N. J. ROSENBROUGH, A. L. FORR AND R. T. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
[7] M. MESELSON AND F. W. STAHL, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 671.
[8] D. NAKADA AND F. J. RYAN, *Microbial Genetics Bull.*, (1960), and in preparation.
[9] C. A. COUGHLIN AND E. A. ADELBERG, *Nature*, 118 (1956) 529.
[10] H. D. BARNER AND S. S. COHEN, *Biochim. Biophys. Acta*, 30 (1958) 112.
[11] D. KANAZIR, H. D. BARNER, J. G. FLAKS AND S. S. COHEN, *Biochim. Biophys. Acta*, 34 (1959) 341.
[12] F. C. NEIDHARDT AND F. GROS, *Biochim. Biophys. Acta*, 25 (1957) 513.
[13] J. HOROWITZ, A. LOMBARD AND E. CHARGAFF, *J. Biol. Chem.*, 233 (1958) 1517.
[14] D. BILLEN, *Biochim. Biophys. Acta*, 34 (1959) 110.
[15] J. TOMIZAWA AND S. SUNAKAWA, *J. Gen. Physiol.*, 39 (1956) 553.
[16] F. M. HAROLD AND Z. Z. ZIPORIN, *Biochim. Biophys. Acta*, 29 (1958) 439.
[17] M. DRACULIC AND M. ERRERA, *Biochim. Biophys. Acta*, 31 (1959) 459.
[18] C. O. DOUDNEY, *J. Bacteriol.*, 79 (1960) 122.

STUDIES WITH PHENAZINE METHOSULFATE: EFFECT ON MITOCHONDRIA — AN ASSAY FOR REDUCED PYRIDINE NUCLEOTIDE COENZYMES*

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SUMMARY

1. Phenazine methosulfate rapidly oxidizes DPNH and TPNH to DPN and TPN, respectively.

2. Phenazine methosulfate can be used in an assay system for microgram quantities of total reduced pyridine nucleotide coenzymes.

3. The results of this assay method in animal tissues agree closely with values previously reported for reduced pyridine nucleotides using enzymic assay methods.

4. Phenazine methosulfate oxidizes the reduced pyridine nucleotides within mitochondria, causes swelling of the mitochondria, and leakage of the coenzymes from mitochondria.

5. Ethylenediaminetetraacetate while inhibiting the swelling and leakage does not inhibit the oxidation. The reduced coenzymes do not appear necessary for the prevention of swelling.

6. Ethanol strongly inhibits the rate of oxidation of DPNH by phenazine methosulfate.

INTRODUCTION

Using appropriate enzymic procedures, it is possible to measure, in microgram quantities, the total reduced and total oxidized pyridine nucleotide coenzymes. Also, the amounts of the individual diphospho- and triphosphopyridine nucleotides may be determined[1,2].

These methods are based on the fact that the coenzymes, in their oxidized states only, form strongly fluorescent products after the addition of certain compounds. By noting, therefore, the increase in fluorescence upon oxidation of the reduced

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Abbreviations: DPN and DPNH, the oxidized and reduced forms of diphosphopyridine nucleotide; TPN and TPNH, the oxidized and reduced forms of triphosphopyridine nucleotide; TCA, trichloroacetic acid; PMS, phenazine methosulfate; ADH, alcohol dehydrogenase; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; IDH, isocitric dehydrogenase.

[*] This is publication number 65 of the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts.

coenzyme, and by reference to a standard, one can determine the amount of reduced coenzyme in a sample.

Using the principles just outlined, a method is reported for the measurement of total reduced pyridine nucleotides utilizing nonenzymic oxidation. The oxidizing agent employed is phenazine methosulfate.

This communication demonstrates the oxidation of microgram quantities of both DPNH and TPNH by PMS. Also, several animal tissues are assayed for total reduced pyridine nucleotides and the results compared with other reported values. This method eliminates the necessity of having to prepare a bacterial enzyme (*Cl. kluyveri*) for the assay.

Effects of phenazine methosulfate on intact mitochondria are also reported.

MATERIALS AND METHODS

DPNH was obtained from Pabst Laboratories and TPNH from Sigma Chemical Company. Yeast alcohol dehydrogenase was purchased from Worthington Biochemical Company. TPN-specific isocitric dehydrogenase was prepared by the method of OCHOA[3]. Phenazine methosulfate was obtained from Sigma Laboratories, and it appears quite stable when kept cold and in the dark.

Spectrophotometric measurements were done in either a Beckman DU or Zeiss spectrophotometer. The fluorometric determinations were performed in a Farrand model A fluorometer or a Coleman electronic photofluorometer using filters as prescribed for thiamine determinations.

Fluorescence of the oxidized pyridine nucleotides was developed by the methylethyl ketone method[4].

Tissues for analysis were homogenized in 5 volumes of sucrose (0.25 *M*)-nicotinamide (0.05 *M*) at 0°. This homogenate was then added to an equal volume of preheated 0.2 *M* Na_2CO_3, and heated in a boiling water bath for 3 min. After centrifugation the supernatant solution was analyzed for total reduced pyridine nucleotides.

Mitochondria were isolated and then suspended in sucrose (0.25 *M*)–nicotinamide (0.05 *M*) at 0°. In these studies, 2.00 ml of mitochondria (1 g of original liver tissue) were added to 0.5 ml 0.25 *M* sucrose and incubated at 28° with shaking. The following substances when used were present in the 0.5 ml sucrose in the following concentrations: potassium phosphate buffer, pH 7.5, 0.1 *M*; phenazine methosulfate, $2 \cdot 10^{-3}$ *M*; EDTA, pH 7.4, $2 \cdot 10^{-2}$ *M*.

Swelling of mitochondria and leakage of pyridine nucleotides were followed as described by KAUFMAN AND KAPLAN[5]. Oxidation of pyridine nucleotides was determined by adding trichloroacetic acid to the whole incubation mixture and developing the fluorescence of an aliquot from the supernatant solution.

RESULTS

Phenazine methosulfate (10^{-5} *M*) in aqueous solution rapidly oxidized DPNH and TPNH to DPN and TPN (Fig. 1). The products of oxidation were identified by their reactions with yeast ADH and isocitric dehydrogenase, respectively. The chemical oxidation and subsequent enzymic reduction were followed by recording the absorption at 340 mμ of the reduced compounds. Absorption due to PMS and to the enzymes was subtracted from the initial figures to give the values recorded here.

Known solutions of DPNH and TPNH in microgram amounts (2–12 μg) were oxidized by PMS, and it was shown that the fluorometric readings were directly proportional to the initial concentration of reduced pyridine nucleotide (Fig. 2).

The effect of increasing ethanol concentrations on DPNH oxidation by PMS was also studied. The reaction mixture contained 0.35 μmole DPNH, 2.85 μmoles phosphate buffer (pH 7.5), and varying amounts of ethanol in a total volume of 3.0 ml. The oxidation was begun by the addition of 0.15 ml $\cdot 10^{-4}$ M PMS and was followed by the decrease in absorption at 340 mμ.

It was found that as the ethanol concentration was increased there was a marked decrease in the observed first order rate constant from 0.43 min^{-1} at 19 % ethanol to 0.19 at 38 %, and to 0.014 at 76 % ethanol.

A number of animal tissues were assayed for total reduced pyridine nucleotides by the PMS method (Table I). From the results it appears that the values agree fairly closely with previously reported values[2]. As shown in Table II, phenazine

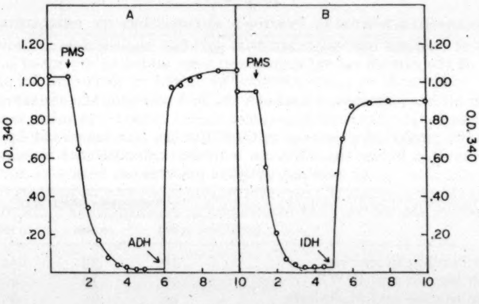

Fig. 1. Oxidation of DPNH and TPNH by phenazine methosulfate and subsequent enzymic reduction. Reactions were run in 1-ml cuvettes. A. Solution contained 0.02 ml DPNH (approx. 6 mg/ml) and 0.98 ml Tris–ethanol (0.5 M with respect to each). At 1 min 0.01 ml of 10^{-3} M PMS was added and at 6 min 0.05 ml of yeast ADH (0.1 dilution) was added. B. Solution contained approx. 0.15 μmole TPNH, 2.5 μmoles sodium isocitrate, 20 μmoles $MgCl_2$, and 0.1 ml 1.0 M phosphate buffer, pH 7.5, in a total volume of 1.0 ml. At 1 min 0.01 ml of 10^{-3} M PMS was added. At 5 min 0.1 ml of isocitric dehydrogenase was added.

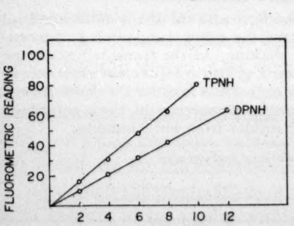

Fig. 2. Oxidation of DPNH and TPNH by phenazine methosulfate. DPNH: Each reaction mixture contained 0.25 ml of 0.1 M Na_2CO_3, 0.20 ml of 1.0 M phosphate buffer, pH 7.5, DPNH at various concentrations, and 0.02 ml $\cdot 10^{-3}$ M PMS in a total volume of 0.9 ml. After 5 min at room temperature, 0.1 ml of 100 % TCA was added. TPNH: Each reaction mixture contained 0.25 ml of 0.1 M Na_2CO_3, 0.20 ml of 1.0 M phosphate buffer, pH 7.5, TPNH at various concentrations, and 0.02 ml $\cdot 10^{-3}$ M PMS in a total volume of 0.9 ml. After 5 min at room temperature 0.1 ml of 100 % TCA was added. For each concentration of DPNH or TPNH two tubes were prepared. In one tube the PMS preceded the TCA, and in the other the TCA was added first. After incubation the fluorescence was developed by the methylethyl ketone method. The fluorometer readings in the accompanying figure represent the differences between the two samples. The fluorometer settings were arbitrary and were not the same for DPNH as for TPNH.

TABLE I

TOTAL REDUCED PYRIDINE NUCLEOTIDE COENZYMES IN SEVERAL RAT ORGANS, EXPRESSED AS mμmoles/g TISSUE

The values reported by the other workers have been converted to mμmoles using 1 μmole, 790 μg. The alkaline extract of the tissue was prepared as described in METHODS. Each assay tube contained 0.25 ml of the alkaline extract, 0.20 ml of 1.0 M potassium phosphate buffer pH 7.5 and 0.02 ml·10^{-3} M PMS in a total volume of 0.9 ml. After incubation for 10 min 0.1 ml of 100% TCA was added. For each sample, a similar tube was prepared in which the addition of TCA preceded PMS. After centrifugation the fluorescence was developed using an 0.5-ml aliquot of the supernatant solution. The difference in fluorometric readings between the 2 tubes, when compared with the readings given by a known amount of DPNH similarly treated, gave a value for reduced pyridine nucleotide.

	No. of samples	*PMS method*	*S d*	*Values reported by* JACOBSON AND KAPLAN[2]	*Values reported by* GLOCK AND MC LEAN[6]
Rat liver	4	532	± 36	455	516
Rat heart	4	298	± 20	234	274
Rat kidney	4	241	± 26	210	337
Rat spleen	4	49	± 19	—	92

TABLE II

OXIDATION OF INTRAMITOCHONDRIAL PYRIDINE NUCLEOTIDES BY PHENAZINE METHOSULFATE

Relative amounts of oxidized intramitochondrial pyridine nucleotides, as shown by fluorometric readings. 2.00 ml of the mitochondrial suspension were added to 0.5 ml of 0.25 M sucrose containing the other compounds in concentrations as stated in METHODS. Incubation was at 28° with shaking. At the times indicated, 0.5 ml of 50% TCA was added to the sample and the volume brought to 5 ml with H_2O. After centrifugation a 1.0-ml aliquot was taken, and the fluorescence was developed by the methylethyl ketone method. For the zero time readings with PMS, it was necessary to add the TCA before the PMS. An increase in fluorescence indicates the appearance of oxidized pyridine nucleotides.

	Fluorometer readings after		
	0 min	*10 min*	*20 min*
Mitochondria in sucrose	20	33	54
In sucrose and EDTA	16	39	46
In sucrose and phosphate	22	91	95
In sucrose, phosphate, and EDTA	17	55	54
In sucrose and PMS	22	82	80
In sucrose, PMS and EDTA	21	78	80

TABLE III

EFFECT OF PHENAZINE METHOSULFATE AND ETHYLENEDIAMINETETRAACETATE ON LEAKAGE OF PYRIDINE NUCLEOTIDES FROM MITOCHONDRIA

Relative amount of leakage is indicated by fluorometric readings. 2.00 ml of the mitochondrial suspension were added to 0.5 ml of 0.25 M sucrose containing the other compounds in concentrations as stated in METHODS. Incubation was at 28° with shaking. At the times indicated the contents of each tube were centrifuged at 0° for 5 min at 12,000 × g. To 1.0 ml of clear supernatant solution was added 0.1 ml 100% TCA. After centrifuging this, 0.5 ml was used for the development of fluorescence by the methylethyl ketone method. Increased fluorescence in the supernatant solution indicates leakage of oxidized pyridine nucleotides from mitochondria.

	Fluorometer readings after		
	0 min	*10 min*	*20 min*
Mitochondria in sucrose	3	9	11
In sucrose and EDTA	3	4	6
In sucrose and phosphate	4	69	80
In sucrose, phosphate, and EDTA	4	9	12
In sucrose and PMS	5	16	65
In sucrose, PMS, and EDTA	4	6	8

methosulfate in the concentration used, oxidized the intramitochondrial reduced pyridine nucleotide coenzymes. EDTA, while inhibiting the oxidation in a phosphate medium, did not inhibit the oxidation by the phenazine compound.

Phenazine methosulfate addition also led to swelling of mitochondria (Fig. 3) and to leakage of the oxidized coenzymes from the mitochondria (Table III). However, both the swelling and leakage were inhibited by EDTA.

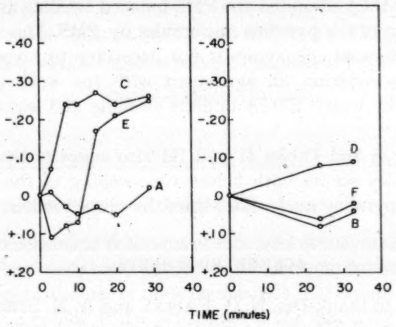

Fig. 3. Effect of phenazine methosulfate and ethylenediaminetetraacetate on swelling of mitochondria. A, in sucrose alone; B, in sucrose and EDTA; C, in sucrose and phosphate; D, in sucrose, phosphate, and EDTA; E, in sucrose and PMS; F, in sucrose, PMS, and EDTA. 2.00 ml of the mitochondrial suspension were added to 0.50 ml 0.25 *M* sucrose containing the other compounds in the concentrations as stated in METHODS. Incubation was at 28° with shaking. At various times 0.05 ml of this suspension was added to 2.95 ml of 0.25 *M* sucrose containing 0.05 *M* EDTA, and the O.D. at 520 mμ was determined. A decrease in O.D. at 520 mμ signified mitochondrial swelling.

DISCUSSION

Phenazine methosulfate (10^{-5} *M*) rapidly oxidized DPNH and TPNH to DPN and TPN. The fact that addition of the appropriate enzyme caused a reappearance of the reduced coenzyme shows that the oxidation products with PMS are DPN and TPN, rather than some further oxidation products such as pyridone compounds.

Fig. 2 shows that the fluorescence developed varied directly with the initial concentration of DPNH or TPNH in the sample being oxidized.

As already observed, when the polarity of the solvent was decreased, by increasing the concentration of ethanol, the rate of oxidation of DPNH by PMS was quite markedly slowed. This would suggest that the transition state in this reaction is more highly charged than the starting compounds.

Since the values in animal tissues by this method of assay compared closely with previously reported values, it would appear that this method is an acceptable and useful one for the oxidation of reduced coenzymes.

Although the mechanisms of mitochondrial swelling are not yet fully understood, there appears to be a relationship to the oxidation–reduction state of the electron transport system. This is shown in the suppression of swelling caused either by anaerobiosis or by a number of inhibitors of electron transport[7-9].

More recently it has been shown that a number of substances stimulating mitochondrial swelling also cause oxidation of pyridine nucleotides and leakage of these coenzymes from mitochondria[10]. It was thought that something further might be learned about these three interrelated phenomena by the use of PMS, which is a potent oxidant of succinic dehydrogenase and of both of the reduced pyridine nucleotide coenzymes.

From the studies with intact mitochondria it appears that phenazine methosulfate is able to oxidize the reduced pyridine nucleotides within mitochondria. It is also of interest that EDTA inhibited the PMS-induced swelling and leakage, without affecting the oxidation of the pyridine nucleotides by PMS. This would suggest that the presence of the reduced coenzymes is not necessary to preserve the structural integrity of the mitochondrion, in agreement with the work of HUNTER *et al.*[9]. However, the means by which EDTA inhibits swelling and leakage remains poorly understood.

Inspection of Fig. 3 and Tables II and III also suggests that the oxidation of the pyridine nucleotides occurs both before the swelling of the mitochondria and before leakage of the pyridine nucleotides from the mitochondria.

ACKNOWLEDGEMENTS

The author would like to thank Drs. N. O. KAPLAN and A. M. STEIN for their interest and suggestions in this work. These investigations were supported in part by a training grant from the National Cancer Institute of the National Institutes of Health (CRT-5033-C1) and also Grant No. CY-3611-C2 of the National Cancer Institute.

REFERENCES

[1] K. B. JACOBSON AND L. ASTRACHAN, *Arch. Biochem. Biophys.*, 71 (1957) 69.
[2] K. B. JACOBSON AND N. O. KAPLAN, *J. Biol. Chem.*, 226 (1957) 603.
[3] S. OCHOA, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. I, Academic Press, New York, 1955, p. 699.
[4] M. M. CIOTTI AND N. O. KAPLAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 897.
[5] B. T. KAUFMAN AND N. O. KAPLAN, *Biochim. Biophys. Acta*, 32 (1959) 576.
[6] G. E. GLOCK AND P. MCLEAN, *Biochem. J.*, 61 (1955) 338.
[7] F. E. HUNTER, Jr., J. DAVIS AND L. CARLAT, *Biochim. Biophys. Acta*, 20 (1956) 237.
[8] A. L. LEHNINGER AND B. L. RAY, *Biochim. Biophys. Acta*, 26 (1957) 643.
[9] F. E. HUNTER, Jr., J. F. LEVY, J. FINK, B. SCHULTZ, F. GUERRA AND A. HURWITZ, *J. Biol. Chem.*, 234 (1959) 2176.
[10] B. T. KAUFMAN AND N. O. KAPLAN, *Biochim. Biophys. Acta*, 39 (1960) 332.

ELECTRONIC STRUCTURE AND BIOCHEMICAL FUNCTION OF FOLIC ACID COENZYMES*

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(Received March 2nd, 1960)

SUMMARY

The two principal biochemical functions of folic acid coenzymes—acting as reversible carriers of one-carbon metabolic groups or as electron donors—are interpreted in terms of the electronic structure of these compounds. The acceptance of the one-carbon unit by N_5 of tetrahydrofolic acid is attributed to the relatively high electronic charge and free valence of that atom. The utility of the isomerisation of the inactive form of the coenzyme, which results from this first reaction, into the active form, in which the one-carbon unit is fixed either at N_{10} or shared by N_5 and N_{10}, may reside in the fact that the departure of the one-carbon unit must be easier in these latter isomers. The electron deficiency on the bond to be ruptured, as measured by its dipositivity is effectively greater when the one-carbon unit is fixed on N_{10} or shared between N_5 and N_{10} than when it is fixed on N_5.

The study of the energies of the molecular orbitals leads to the conclusion that tetrahydrofolic acid must be a good electron donor. The study of the electronic structure of folic acid, leads to the prediction, which seems to be in agreement with experiment, that its reduction should be easier at the 7–8 bond than at the 5–6 bond. The possible reduction product N_5–N_8 dihydrofolic acid presents the unusual property of having an antibonding highest filled orbital and should consequently resist further reduction since it is a particularly good electron donor.

INTRODUCTION

Folic acid (I), a vitamin of the B group, is converted *in vivo* through a series of enzymic transformations into a coenzyme form, which is an essential reversible carrier of a one-carbon metabolic group, at the oxidation level of formate or formaldehyde[1]. The class of enzymes which utilise the folic acid coenzymes are known under the general name of "pteroproteins".

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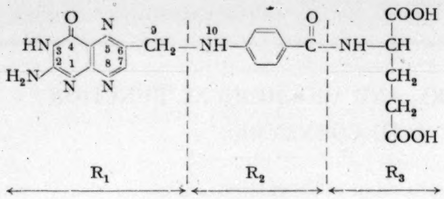
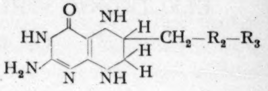


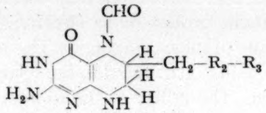
I. Folic acid (F)



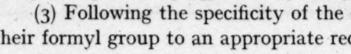
II. Tetrahydrofolic acid (FH_4)

The coenzyme form of folic acid (symbol F) is the 5,6,7,8-tetrahydrofolic acid (II) (symbol FH_4). The general pattern of its biological function as a carrier of one-carbon unit, *e.g.* the formyl unit (symbol f), may be resolved into three stages: [2–4]

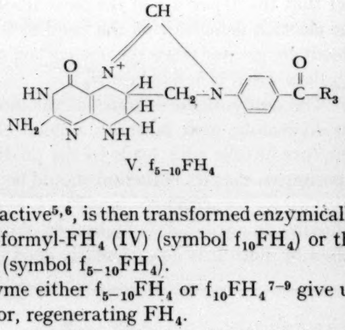
(1) The acceptance of the one-carbon group from a donor. The one-carbon unit becomes attached to N_5 of the coenzyme giving rise to e.g. 5-formyl-FH_4 (III) or folinic acid (symbol f_5FH_4)*.



III. f_5FH_4



IV. $f_{10}FH_4$



V. $f_{5-10}FH_4$

(2) This form of the coenzyme, which is inactive[5,6], is then transformed enzymically into an active form which may be either 10-formyl-FH_4 (IV) (symbol $f_{10}FH_4$) or the bridge compound N_5,N_{10}-methenyl FH_4 (V) (symbol $f_{5-10}FH_4$).

(3) Following the specificity of the enzyme either $f_{5-10}FH_4$ or $f_{10}FH_4$[7–9] give up their formyl group to an appropriate receptor, regenerating FH_4.

Among the most important reactions catalyzed in this way by the folic acid coenzymes are some essential steps in the *de novo* synthesis of the purines of the nucleic acids, especially the incorporation of C_2 and C_8 into the skeletons of these bases[10–13].

A second important biochemical function of the folic acid coenzymes is to act as electron donors, particularly in the formation of the methyl groups of choline and thymine from formaldehyde[14–16]. The reaction necessitates the oxidation of FH_4 to FH_2.

In connection with this aspect of the biochemical role of folic acid, there is also the problem of the structure and significance of the dihydrofolic acids (symbol FH_2) which represent the possible intermediate steps in the reduction of F to FH_4 or the reoxidation of FH_4 to F. The information available in this field is complex and does not lead to any straightforward conclusion. Thus, *e.g.* FUTTERMAN[17] has purified an enzyme system which catalyzes both the reduction of F to FH_2 (TPNH dependent reaction) and the reduction of FH_2 to FH_4 (DPNH or TPNH dependent reaction), a result which suggests a two steps biosynthesis of FH_4 with an intermediate formation of an FH_2. On the other hand WRIGHT *et al.*[18–20] who have studied the enzymic reduction of F by folic reductase purified from *C. Sticklandii* according to the reaction

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* In ATP requiring enzymic reactions, the formyl group is transferred directly to position 10 of the 10-phosphorylated FH_4. These reactions will be discussed in a subsequent paper.

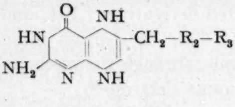
$$F + CoASH + \text{pyruvate} \longrightarrow FH_2 + CoASCOCH_3 + CO_2$$

(in which pyruvate is the electron donor) have not been able to reduce further the FH_2 which is formed to FH_4; the F–FH_2 couple functions as an oxido-reduction system, F being the electron acceptor and FH_2 the electron donor. Nevertheless, GREENBERG *et al.*[15,21] showed the existence in mammalian tissue (rat thymus) of a reversible enzymic reduction of FH_2 to FH_4

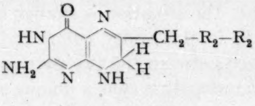
$$FH_4 + DPN^+ \rightleftarrows FH_2 + DPNH$$

A similar reaction, in avian and mammalian livers, mediated by a TPN-linked dehydrogenase, has been observed by OSBORN AND HUENNEKENS[22].

The question occurs then, of course, as to the structure (or the structures) of the dihydrofolic acid (or acids) which are formed as intermediates in these different transformations of F to FH_4 or *vice-versa*. PETERS AND GREENBERG[14] suggest as the active forms of FH_2, obtained by enzymic oxidation of FH_4, either N_5–N_8 FH_2 (VI) or C_7–N_8 FH_2 (VII). OSBORN AND HUENNEKENS[22], more exclusive, estimate that the enzymic reoxidation of FH_4 yields C_7–N_8 FH_2. BLAKLEY[23,24] who observed that FH_2, obtained by enzymic or chemical reduction of F, yields the 5-hydroxymethyl derivative through the action of HCHO, deduces from this result that the reduction obviously involves position 5; but the results do not permit it to be ascertained whether this takes place at positions 5–8 or 5–6[25]. The question is thus unsettled.



VI. N_5–N_8 FH_2



VII. C_7–N_8 FH_2

The present investigation was undertaken with the aim of contributing towards the elucidation of the mechanism of action of the folic acid coenzymes by establishing in particular the factors responsible for their functioning as carriers of one-carbon units and for their involvement in hydrogen transfer reactions. It also aimed towards the elucidation of the structure and properties of the different possible dihydro intermediates between F and FH_4. Finally, a further aim of this investigation was the possible application of the results to the study of the mechanism of action of folic acid antimetabolites, in particular of antimetabolites which, like aminopterin or amethopterin, are active in cancer chemotherapy[26–29]. Their activity seems to be related to the blocking of the enzymic reduction of F to FH_4, and it may consequently be hoped that the elucidation of the reactions involved in these transformations may throw light on the mechanism of action of the antimetabolites. However, this problem of antimetabolites will be treated in a separate publication.

METHODS

The method employed for the study of the electronic structure of the compounds involved is the L.C.A.O. approximation of the molecular orbital method of quantum chemistry[30], with our usual appropriate parameters[31]. The only new case was that of the quaternary nitrogen of $f_{5-10}FH_4$. Previously, we have used for the Coulomb integral of the quaternary nitrogen of DPN^+ the value $\alpha_{N^+} = \alpha_C + 2\beta$[32]. As the positive charge in $f_{5-10}FH_4$ resonates between two nitrogens, N_5 and N_{10}, the previous value of α_{N^+} seemed to be too great for the present case and, consequently, a reduced value of $\alpha_N = \alpha_C + 1.5\beta$ has been assumed for both the N_5 and N_{10} of $f_{5-10}FH_4$.

Folic acid, its derivatives and coenzymes may, in principle, manifest keto–enol tautomerism. Since a study of hydroxypteridines has shown that these molecules exist essentially in the keto form[33], we have considered only this form for all the compounds discussed in this paper.

The structure of folic acid is obviously complex and is composed of three fragments: the pteridine nucleus (R_1), the *para*aminobenzoic system (R_2) and the glutamic acid residue (R_3). Of this last fragment, only the N–H group has been introduced into the calculations. The remaining portion of the residue is separated from R_1 and R_2 by a saturated carbon and consequently does not participate in the general electronic conjugation.

Folic acid has consequently been considered as a system composed of a 2,amino-4,hydroxypteridine, united through the hyperconjugation of a CH_2 group to a *para*-aminobenzoylamine. It is thus a unique system of 28 π electrons.

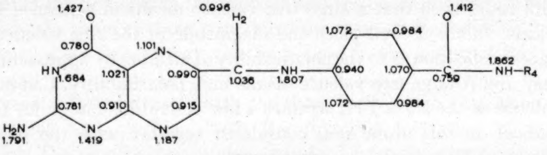
FH_4 on the contrary, because of the saturation of C_6, has to be considered as composed of two separated units, which are the hydrogenated pteridine nucleus (14 π electrons) and the *para*aminobenzoic acid fragment (12 π electrons). No interaction of mobile electrons exist, at a first approximation, between these two portions of the coenzyme. The situation is similar in the formylated derivatives f_5FH_4 and $f_{10}FH_4$. It is only in the bridge compound $f_{5-10}FH_4$ that direct π electron interaction is established between the two portions of the molecule, through the methenyl bridge. This last molecule is thus a unique system of 26 mobile electrons[34].

RESULTS AND DISCUSSION

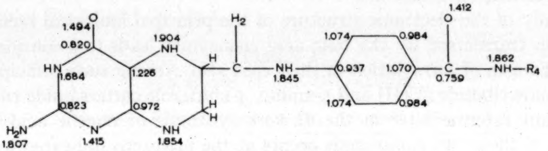
Folic acid coenzymes as one-carbon unit carriers

As one-carbon unit acceptors: Following the excellent definition proposed by Koshland[35], group transfers may be considered as enzymic substitution reactions. Consequently one may plausibly seek to interpret them in terms of structural characteristics succesfully employed for the interpretation of substitution reactions in general. These characteristics may be looked for in the first place in the electronic properties of the atoms constituing the conjugated substrate which undergoes the substitution. There are two principal properties to be considered: the electronic charges and the free valences of the atoms[30, 36].

Fig. 1 represents the distribution of the electronic charges in folic acid and in tetrahydrofolic acid. Limiting ourselves to the problem considered in this section and examining the charge distribution in tetrahydrofolic acid, which is the one-carbon unit acceptor, we may obviously draw the following first conclusion: the one carbon unit is being accepted by the hydrogen carrying N atom of FH_4 which has the greatest charge of the mobile or π electrons.


1.427
O
0.780
1.101
N
0.996
H2
1.072
0.984
1.412
O
HN
1.684
1.021
0.990
C
1.036
NH
1.807
0.940
1.070
C
0.759
1.862
NH—R4
0.781
0.910
0.915
1.072
0.984
H2N
1.791
N
1.419
N
1.187

Folic acid (I)


1.494
O
0.820
1.904
NH
H2
H
C
NH
1.845
1.074
0.984
1.412
O
HN
1.684
1.226
0.937
1.070
C
0.759
1.862
NH—R4
0.823
0.972
H
H
1.074
0.984
H2N
1.807
N
1.415
NH
1.854

Tetrahydrofolic acid (II)

Fig. 1. Electronic charges (see text).

As is well known, when an NH group is incorporated into a conjugated system, the "lone pair" of electrons situated on this N atom effectively participates in the conjugation (the delocalisation) of the π electrons of the system. The result is a discharge of those nitrogen atoms, a fraction of the electrons of the lone pair migrating into the conjugated ring. Because of this electronic delocalisation these N atoms keep less than two electrons: they carry, consequently, a certain formal positive charge which represents the difference between the 2 electrons which the N atom would possess in the absence of any conjugation and that of its effective electronic charge[37]. Thus *e.g.* the formal positive charge of the N_5 of FH_4 is $2e - 1.904e = 0.096e$ and it may be observed that it is the smallest of the formal positive charges carried by the NH groups in FH_4. It is thus the N_5 of the FH_4 skeleton which out of all similar atoms conserves the greatest fraction of its lone pair and it is on this atom that the one-carbon group is fixed.

As a matter of fact the N_5 of FH_4 has also another outstanding property. Thus Fig. 2 represents the values of the free valences of all the hydrogen-carrying nitrogens of FH_4 (these free valences being obtained by following the usual procedure of substracting from a constant, taken in this case to be equal to 1.42, the sum of the mobile

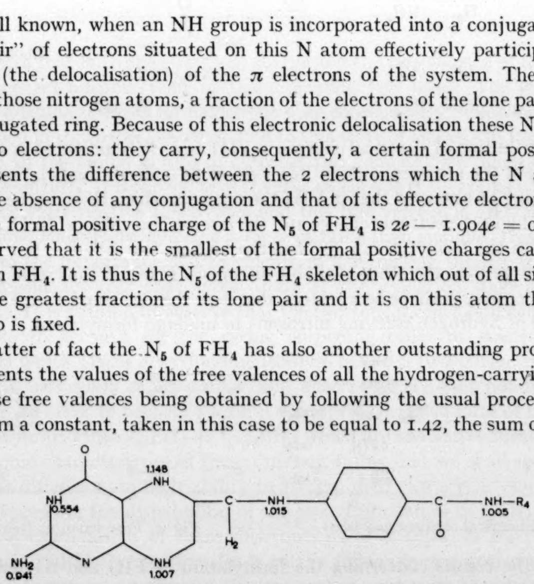
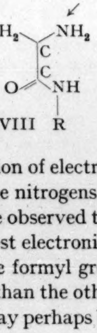

O
1.148
NH
NH
0.554
C
NH
1.015
C
NH—R4
1.005
H2
O
NH2
0.941
N
NH
1.007

Fig. 2. Free valences (see text).

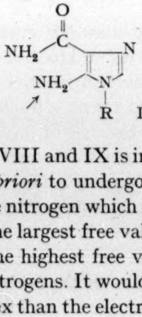
bond orders of the bonds adjacent to each N) and it may be observed that it is N_5 again which also has the greatest free valence. Consequently, it may also be said that the one carbon unit is accepted by the NH group of FH_4 whose nitrogen has the greatest free valence.

The significance of this second correlation may be two-fold. Thus, in the first place, the free valence of an atom is a direct measure of its ability to undergo substitution reactions with radicals so that a large free valence means in general a high affinity for free radicals. In the second place the magnitude of the free valence gives also, generally, a good indication as to the polarisability of an atom by approaching reagents, whatever they are. A large free valence means high polarisability. Consequently, the large free valence of the N_5 of FH_4 signifies a low activation energy for the substitution of a radical on this atom and, considered together with the relatively high electronic charge of that atom, signifies also a low activation energy for the electrophilic substitution of that atom. The formylation reaction of FH_4 at N_5 may thus use one or both of these mechanisms.

The study of the electronic structure of the principal biological receivers of the formyl group transferred by the folic acid coenzymes leads to a consideration of a similar mechanism of formylation in their case also. Among such principal receivers are glycinamide ribotide (VIII) and 5-amino, 4-imidazole carboxamide ribotide (IX), two important intermediates in the *de novo* synthesis of nucleic acids[10, 38, 39]. The formylation of these two compounds occurs at the nitrogens indicated by arrows on the formulas.



VIII



R = Ribose-phosphate

IX

The distribution of electrical charges in VIII and IX is indicated in Fig. 3 and the free valences of the nitrogens susceptible *a priori* to undergo formylation are given in Fig. 4[40]. It may be observed that in VIII the nitrogen which receives the formyl group has both the highest electronic charge and the largest free valence. In IX, the nitrogen which receives the formyl group also has the highest free valence, but has a smaller electronic charge than the other available nitrogens. It would thus seem plausible that the free valence may perhaps be a better index than the electronic charge for measuring the aptitude of hydrogen carrying nitrogens to undergo formylation.

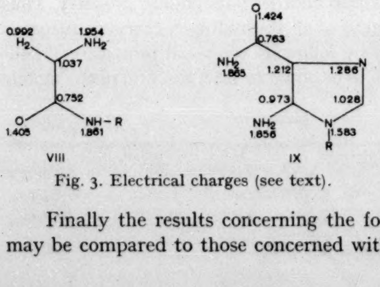


Fig. 3. Electrical charges (see text).

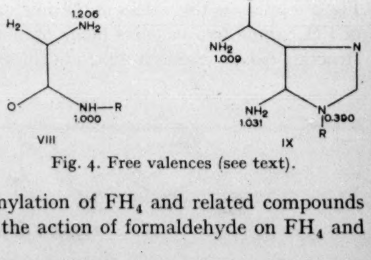


Fig. 4. Free valences (see text).

Finally the results concerning the formylation of FH_4 and related compounds may be compared to those concerned with the action of formaldehyde on FH_4 and on some other fundamental biochemicals[1]. Thus FH_4 serves as a coenzyme for the enzymic system which synthetizes serine from glycine and formaldehyde. The first step in this synthesis consists in a non-enzymic combination of formaldehyde with FH_4[41]. According to BLAKLEY[41,42] the reaction leads probably to the formation of N_5–N_{10}-methylenetetrahydrofolic acid, through the intermediate formation of N_5-hydroxymethyltetrahydrofolic acid. The reaction of formaldehyde with the N_5 of FH_4 is thus probably the first step in the reaction and the electronic factors responsible for it are certainly the same as those considered for enzymic formylation.

The role of these factors may also be noticed in the action of formaldehyde on the purine and pyrimidine bases of the nucleic acids. According to FRAENKEL-CONRAT[43] formaldehyde interacts with the three bases which possess an NH_2 group, adenine, guanine and cytosine, with the probable formation of a Schiff base ($—N = CH_2$). The relative reactivities as judged from the approximate increase in the u.v. absorption are in the order: adenylic acid > cytidylic acid > guanylic acid.

This may be compared with the electronic characteristics of the nitrogens of the amino group of the bases[44] (Table I).

TABLE I

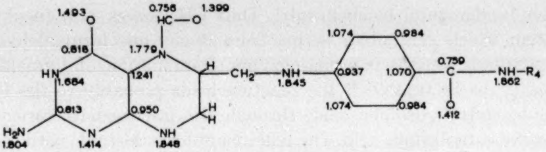
PROPERTIES OF THE NH_2 GROUPS

Compounds	*Electrical charge on N of NH_2*	*Free valence of N of NH_2*
Adenine	1.310	0.960
Cytosine	1.803	0.944
Guanine	1.803	0.933

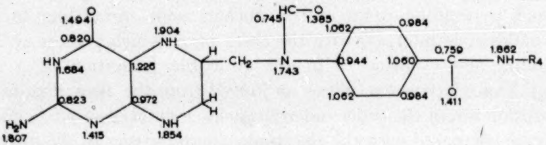
We observe that both the electronic charge and the free valence of the N atom of NH_2 are larger in adenine than in the remaining two bases. The great relative reactivity of adenine towards formaldehyde may consequently, following the general ideas developed in this paper, be attributed to this state of affairs. On the other hand, the nitrogens of the amino groups of cytosine and guanine have identical electrical charges. Consequently, it is the value of the free valence of these atoms which must be responsible for their relative reactivities and this free valence is greater in cytosine than in guanine. Effectively, cytosine is more reactive than guanine. This result could again point to the importance of free valence in determining the reactivity of heterocyclic nitrogens towards one carbon units at the oxidation stage of formate or formaldehyde. It is striking to observe that the theoretical considerations developped here are able to account for the rather surprising order of reactivity towards formaldehyde which places a pyrimidine ribotide between two purine ribotides.

Folic acid coenzymes as one-carbon unit donors: f_5FH_4 does not function as a formyl donor. In order to become a formyl donor, this inactive form of the coenzyme must be transformed into $f_{10}FH_4$ or $f_{5-10}FH_4$ which are the active forms. It is again in the electronic characteristics of these different forms that we shall seek the explanation for the differences in their ability to give up their extracyclic one-carbon unit.

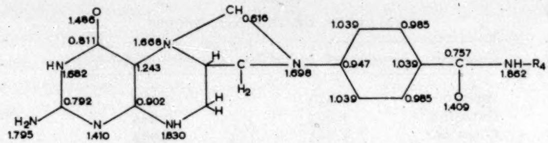
Fig. 5 represents the distribution of the electrical charges in f_5FH_4, $f_{10}FH_4$ and $f_{5-10}FH_4$. The examination of those distributions of charges, in relation with the problem considered in this section, leads to the following principal conclusion: the ability of the coenzyme to give up the one-carbon unit may be related to the di-


1.493
O
0.756
1.399
HC—O
0.816
1.779
N
HN
1.684
1.241
H
CH₂
NH
1.845
1.074
0.984
0.937
1.070
0.759
C
NH—R₄
1.862
0.813
0.950
H
H
1.074
0.984
O
1.412
H₂N
1.804
N
1.414
NH
1.848

f₅· FH₄ (III)


1.494
O
0.745
HC—O
1.385
0.820
1.904
NH
HN
1.684
1.226
H
CH₂
N
1.743
1.062
0.984
0.944
1.060
0.759
C
1.862
NH—R₄
0.823
0.972
H
H
1.062
0.984
O
1.411
H₂N
1.807
N
1.415
NH
1.854

f₁₀· FH₄ (IV)


1.486
O
CH
0.616
0.811
1.668
N
HN
1.682
1.243
H
C
H₂
N
1.698
1.039
0.985
0.947
1.039
0.757
C
NH—R₄
1.862
0.792
0.902
H
H
1.039
0.985
O
1.409
H₂N
1.795
N
1.410
NH
1.830

f₅₋₁₀· FH₄(V)

Fig. 5. Electrical charges (see text).

positivity of the N–C bond which has to be ruptured. This dipositivity is greater in the active forms of the coenzyme than in the inactive one.

The term "dipositivity" may need an explanation[37]. It can be observed that both the nitrogen atom of FH_4 upon which the formyl group is fixed and the carbon atom of this group are electron deficient. They both carry formal positive charges which may be calculated by subtracting from 2 the electronic charge on the nitrogen atom and from 1 the electronic charge on the carbon atom. The "dipositivity" of the bond refers to the presence of such formal positive charges on both of the atoms of the bond. In $f_{5-10}FH_4$ there are, naturally, two such bonds around the one-carbon unit. Table II contains an evaluation of this dipositivity of the bond to be ruptured for the different forms of the formylated coenzyme.

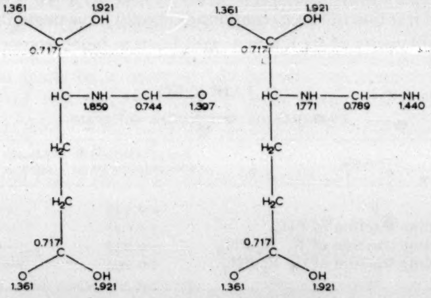
TABLE II

DIPOSITIVITY OF BONDS

Compound	*Formal positive charge on the N atom carrying the formyl group*	*Formal positive charge on the C atom of the formyl group*	*Dipositivity of the N–C bond*
f_5FH_4	0.221	0.244	0.465
$f_{10}FH_4$	0.257	0.255	0.512
$f_{5-10}FH_4$	N_5 = 0.332 N_{10} = 0.302	0.384	0.716 0.686

As stated before this dipositivity is appreciably greater in the active forms than in the inactive one and it seems, consequently, plausible to admit that it is the value of this dipositivity which is responsible, at least to some extent, for the relative ability of the different forms to give up the one-carbon unit. A larger dipositivity represents greater electronic repulsion in the bond under consideration, so that the larger its electronic deficiency the weaker the bond. This conclusion is substantiated by similar results obtained recently in the study of the electronic structure of the energy-rich phosphates[45] and the electronic structure of the principal types of biochemical bonds undergoing enzymic hydrolysis[37]. In both cases the dipositivity of the ruptured bonds was an essential feature of their structure. Thus the isomerisation of the inactive f_5FH_4 into the active $f_{10}FH_4$ or $f_{5-10}FH_4$ leads to a more economically functioning form of the coenzyme. As a confirmation of this point of view it may be useful to quote the results of the determination of the free energies of the (non-enzymic) hydrolysis of the compounds cited. The relative free energies of hydrolysis at pH 7 stand in the order $f_{5-10}FH_4 > f_{10}FH_4 > f_5FH_4$[1] a result which points out at the same time the greater lability of the one-carbon unit in $f_{5-10}FH_4$ or $f_{10}FH_4$ than in f_5FH_4 and the important role of the degree of electron deficiency of a bond for the ease of its hydrolysis. Another striking observation in this field is that, in purified extracts of *Clostridium cylindrosporum*, $f_{5-10}FH_4$ is opened selectively to $f_{10}FH_4$ by the enzyme cyclohydrolase. The bond which is hydrolysed is the most electron deficient of the two susceptible ones, a result which is again in agreement with the elements of the general theory of enzymic hydrolysis as proposed by B. AND A. PULLMAN.

In this field, again, it may be useful to investigate whether the one-carbon unit donor properties of other important biochemical substances may also be related to the same factor. That this seems to be essentially the case may be concluded from the important observation that while formiminoglutamic acid, a degradation product of histidine[2, 46–48], is an important formylation agent for the enzymic synthesis of the active coenzyme, formylglutamic acid is unable, in the same conditions, to give up its one-carbon unit. The distribution of electrical charges in these two substances is given in Fig. 6 and the dipositivity of the susceptible bonds evaluated in Table III.



Formylglutamic acid Formiminoglutamic acid

Fig. 6. Electronic charges (see text).

It can be seen that the bond maintaining the one-carbon unit is more dipositive in the active formiminoglutamic acid than in the inactive formylglutamic acid.

TABLE III

DIPOSITIVITY OF BONDS

Compound	Formal positive charge on the N atom carrying the formyl group	Formal positive charge on the C atom of the formyl group	Dipositivity of the N–C bond
Formylglutamic acid	0.141	0.256	0.397
Formiminoglutamic acid	0.229	0.211	0.440

Finally, one may consider, as a complementary factor which favors $f_{10}FH_4$ as the active form of the one-carbon unit donor, the variation of the resonance energy of the coenzyme which accompanies the departure of the formyl group. The departure is, as far as the folic acid coenzyme is concerned, accompanied by a decrease in its resonance energy. This decrease is nevertheless smaller for the departure of the formyl group from the N_{10} of FH_4 than from its N_5, the corresponding resonance energy variations being, respectively, 0.371 β and 0.400 β.

Electron (hydrogen) donor and acceptor properties of di- and tetrahydrofolic acids

As shown recently by B. AND A. PULLMAN the electron donor and acceptor properties of biochemicals may be satisfactorily accounted for in terms of the energies of their highest filled and lowest empty molecular orbitals[49]. The same considerations will now be applied to the theoretical investigation of these properties in the di- and tetrahydrofolic acids, in connection with the different experimental observations in this field quoted briefly in the introducting remarks of this paper.

Table IV contains the energies of the highest occupied molecular orbital (h.o.m.o.) and the lowest empty molecular orbital (l.e.m.o.) of F and of the pteridine fraction of FH_4 and of the two isomeric FH_2's: the N_5–N_8FH_2 and the C_7–N_8FH_2. In the hydrogenated derivatives of F, it is this pteridine portion of the molecule which is effectively the essential center of the electron transfer properties and, as it is to a large extent isolated from the remaining parts of the compound, it is the only one which needs to be considered here. (For information it may be quoted that the *para*aminobenzamide central fraction of these compounds should have negligible electron transfer capacities, the coefficients of its h.o.m.o. and l.e.m.o. being respectively +0.555 and −1.)

TABLE IV

ENERGIES OF MOLECULAR ORBITALS

Compound	Energy of the highest filled molecular orbital	Energy of the lowest empty molecular orbital
F	+0.446	−0.648
The pteridine fraction of FH_4	+0.048	−1.071
The pteridine fraction of N_5–N_8 FH_2	−0.237	−0.927
The pteridine fraction of C_7–N_8 FH_2	+0.293	−0.748

The results of Table IV indicate that:

(a) Folic acid itself should be a moderate electron donor or acceptor, according to the conditions. Its moderate electron acceptor properties have been demonstrated recently by FUJIMORI who showed that this compound was able to form to some extent charge transfer complexes with tryptophan (acting as an electron donor)[50]. Nevertheless this ability was much smaller for folic acid than, *e.g.*, for riboflavin.

(b) The distribution of the energy levels in FH_4 is profoundly different from that in the parent F. FH_4 appears to be an excellent electron donor, a property confirmed by its ready reoxidation to dihydrofolic acid, folic acid and degradation products[42]. Its electron acceptor properties are bound to disappear, a conclusion again confirmed by its inability to form a charge transfer complex with tryptophan[50].

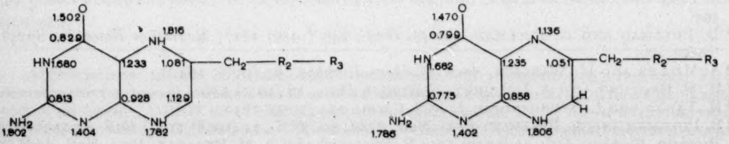
(c) The two dihydrofolic acids considered should be electron donors, but in this respect the electron donor properties of N_5–N_8FH_2 should be particularly outstanding. In fact, this molecule manifests the unusual property, first discovered by B. AND A. PULLMAN for the case of the reduced form of the riboflavin coenzymes, of possessing an antibonding highest occupied orbital[49]. Such a property must be associated with an usually low ionization potential and extremely pronounced electron donor capacities.

In view of these results it seems tempting to postulate that it is the formation of this N_5–N_8FH_2 which occurs in the course of the reduction of F at conditions at which it does not appear to be possible to continue the reduction till the formation of FH_4. The unusually strong electron donor properties of this compound preclude such a continuation and lead to a reversible dehydrogenation.

Correlatively, the formation of the C_7–N_8 FH_2 may be postulated as an intermediate in the reductions which end in FH_4.

We have omitted from our considerations the third possible isomer N_5–C_6FH_2 both because the experimental evidence seems to preclude its formation and because such a preclusion is justified on theoretical grounds. Effectively, the examination of the appropriate electronic index of folic acid leads to the conclusion that of the two reductions—the saturation of either the N_5–C_6 bond or that of the C_7–N_8 bond—the second one is far more probable. Thus, as the two reductions represent addition reactions to a bond, and most probably atomic addition reactions, the relative ease of the reduction at these two similar bonds will be determined essentially by (a) the value of the mobile order of the bond and by (b) the free valences[30] of the reactive atoms, in the present case especially, of the N_5 and N_8 atoms. The bond orders of the N_5–C_6 and C_7–N_8 bonds of F are, respectively, 0.656 and 0.683 and the free valences of N_5 and N_8 0.120 and 0.158[34]. The reduction of folic acid at the C_7–N_8 bond appears, consequently, to be more probable than its reduction at the N_5–C_6 bond. Moreover, this reduction also leads to a more stable isomer, the resonance energies of the pteridine residues of the two dihydrofolic acids being, respectively, 3.197 β for C_7–N_8FH_2 and 3.041 β for the N_5–C_6 isomer. It may be useful to add that the resonance energy for the N_5–N_8FH_2 is 3.525 β.

We reproduce in Fig. 7 the distribution of electrical charges in the two probable FH_2 forms.



N_5-N_8 FH_2 (VI) C_7-N_8 FH_2 (VII)

Fig. 7. Electrical charge (see text).

REFERENCES

[1] F. M. Huennekens and M. J. Osborn, *Advances in Enzymol.*, 21 (1959) 369.

[2] A. Miller and H. Waelsch, *J. Biol. Chem.*, 228 (1957) 400.

[3] R. Koehler, L. Goodman, J. Degraw and B. R. Baker, *J. Am. Chem. Soc.*, 80 (1958) 5779.

[4] H. Tabor and L. Wyngarden, *Federation Proc.*, 18 (1959) 336.

[5] C. A. Nichol, A. M. Anton and S. F. Zakrzewski, *Science*, 121 (1955) 275.

[6] H. P. Broquist, *Ann. Rev. Biochem.*, 27 (1958) 285.

[7] S. C. Hartman and J. M. Buchanan, *J. Biol. Chem.*, 234 (1959) 1812.

[8] J. R. Totter, *Ann. Rev. Biochem.*, 26 (1957) 181.

[9] L. Jaenicke, *Biochim. Biophys. Acta*, 17 (1955) 588.

[10] J. M. Buchanan, J. G. Flaks, S. C. Hartman, B. Levenberg, L. N. Lukens and L. Warren, *The Chemistry and Biology of Purines*, Ciba Foundation Symposium, Churchill Ltd., London, 1957, p. 233.

[11] G. R. Greenberg and L. Jaenicke, *The Chemistry and Biology of Purines*, Ciba Foundation Symposium, Churchill Ltd., London, 1957, p. 204.

[12] J. G. Flaks, M. J. Erwin and J. M. Buchanan, *J. Biol. Chem.*, 229 (1957) 603.

[13] J. M. Buchanan and S. C. Hartman, *Advances in Enzymol.*, 21 (1959) 199.

[14] J. M. Peters and D. M. Greenberg, *J. Am. Chem. Soc.*, 80 (1958) 6679.

[15] G. K. Humphreys and D. M. Greenberg, *Arch. Biochem. Biophys.*, 78 (1958) 275.

[16] D. M. Greenberg and G. K. Humphreys, *Federation Proc.*, 17 (1958) 234.

[17] S. Futterman, *J. Biol. Chem.*, 228 (1957) 1031.

[18] M. L. Anderson and B. E. Wright, *J. Am. Chem. Soc.*, 79 (1957) 2027.

[19] B. E. Wright and M. L. Anderson, *Biochim. Biophys. Acta*, 28 (1958) 370.

[20] B. E. Wright, M. L. Anderson and E. L. Herman, *J. Biol. Chem.*, 230 (1958) 271.

[21] D. M. Greenberg and G. K. Humphreys, *Federation Proc.*, 17 (1958) 234.

[22] M. J. Osborn and F. M. Huennekens, *J. Biol. Chem.*, 233 (1958) 969.

[23] R. L. Blakley, *Biochem. J.*, 65 (1957) 331.

[24] R. L. Blakley, *Biochem. J.*, 72 (1959) 707.

[25] R. L. Blakley, *Current Trends in Heterocyclic Chemistry*, Butterworths Scientific Publications, London, 1958, p. 140.

[26] T. H. Jukes, *Federation Proc.*, 12 (1953) 633.

[27] D. A. Goldthwait, *The Leukemias*, Henry Ford Hospital International Symposium, Academic Press, New York, 1957, p. 555.

[28] M. J. Osborn, M. Freeman and F. M. Huennekens, *Proc. Soc. Exptl. Biol. Med.*, 97 (1958) 429.

[29] S. F. Zakrzewski and C. A. Nichol, *Biochim Biophys. Acta*, 27 (1958) 425.

[30] B. Pullman and A. Pullman, *Les Théories Électroniques de la Chimie Organique*, Masson Ed., Paris, 1952.

[31] B. Pullman and A. Pullman, *La chimiothérapie des cancers et des leucémies*, CNRS International Symposium, Paris, 1958, p. 201; *Bull. soc. chim. France*, (1958) 766; *Compt. rend.*, 246 (1958) 611.

[32] B. Pullman and A. Pullman, *Proc. Natl. Acad. Acad. Sci. U.S.*, 45 (1959) 136.

[33] S. F. Mason, *The Chemistry and Biology of Pteridines*, Ciba Foundation Symposium, Churchill Ltd., London, 1954, p. 74.

[34] B. Pullman, *Compt. rend.*, 246 (1958) 3292.

[35] D. E. Koshland in W. D. McElroy and B. Glass, *Symposium on The Mechanism of Enzyme Action*, Baltimore, 1954, p. 608.

[36] B. Pullman and A. Pullman, *Progress in Org. Chem.*, 4 (1958) 31.

[37] B. Pullman and A. Pullman, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 1572.

[38] D. A. Goldthwait, R. A. Peabody and G. R. Greenberg, *J. Am. Chem. Soc.*, 76 (1954) 5258.

[39] J. G. Flaks, L. Warron and J. M. Buchanan, *J. Biol. Chem.*, 228 (1957) 215.

[20] B. Pullman and A. Pullman, *Compt. rend.*, 246 (1958) 1613.

[41] R. L. Blakley, *Biochem. J.*, 72 (1959) 707.

[42] R. L. Blakley, *Biochem. J.*, 65 (1957) 331.

[43] H. Fraenkel-Conrat, *Biochim. Biophys. Acta.*, 15 (1954) 307.

[44] A. Pullman and B. Pullman, *Bull. soc. chim. France*, (1958) 766; *Bull. soc. chim. France*, (1959) 594.

[45] B. Pullman and A. Pullman, *Compt. rend.*, 249 (1959) 1827; *Radiation Research*, Suppl. 2 (1960) 160.

[46] A. Miller and H. Waelsch, *Arch. Biochem. Biophys.*, 63 (1956) 263.

[47] H. P. Broquist and A. L. Luhby, *Federation Proc.*, 16 (1957) 159.

[48] H. Tabor and L. Wyngarden, *J. Biol. Chem.*, 234 (1959) 1830.

[49] B. Pullman and A. Pullman, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 1197; *Ibid.*, 45 (1959) 136; *Biochim. Biophys. Acta*, 35 (1959) 535; B. Pullman and A. M. Perault, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 1476.

[50] E. Fujimori, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 133.

NITRATE REDUCTASE OF NITRATE RESPIRATION TYPE FROM *E. COLI*

I. SOLUBILIZATION AND PURIFICATION FROM THE PARTICULATE SYSTEM WITH MOLECULAR CHARACTERIZATION AS A METALLOPROTEIN*

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SUMMARY

A particulate nitrate reductase system which included cyt. b_1 as an intermediary carrier from formate or DPNH to nitrate was prepared from *E. coli* cells grown anaerobically in the presence of nitrate; this system possessed remarkably high activity of nitrate reductase of the nitrate respiration type. The particulate preparation, devoid of activities suggestive of not only reduced pyridine nucleotides oxidases but also dehydrogenases except for formate was, as far as has been tested, functionally characterized by its highly active formate–nitrate reductase system. Nitrate reductase, the terminal enzyme of the particulate system, was solubilized by cold alkaline incubation after heat treatment and then purified to a homogeneous state. The enzyme was shown to have a molecular weight of a million and to contain one atom of bound molybdenum and about 40 atoms of bound iron per molecule but no bound flavin. A difference spectrum (oxidized minus reduced) having a broad peak at 445–450 mμ disappeared rapidly on the addition of nitrate with simultaneous production of nitrite. The turnover number of the enzyme was estimated to be about $7.0 \cdot 10^3$ moles/sec from reduced methylviologen, the most effective electron donor. Examination of the physiological donors for the purified enzyme showed that reduced forms of pyridine nucleotides were never effective and flavin derivatives were little effective. Data which suggested that reduced cyt. b_1 would be a more favorable donor for nitrate reductase were presented. The homogeneously purified enzyme seems to function as a terminal enzyme in a nitrate-respiring system *in vivo* and may be regarded as an anaerobic variant of terminal oxidases. Biochemical comparison between *E. coli* enzyme (nitrate respiration type) and *Neurospora* enzyme (nitrate assimilation type) as well as their electron-transferring mechanisms have been discussed.

Abbreviations: nitrate reductase, NaR; cytochrome, cyt.; formate dehydrogenase, FDH; reduced methyl or benzyl viologen, MVH or BVH; methylene blue, Mb; reduced di- or triphosphopyridine nucleotide, DPNH or TPNH; flavinadenine dinucleotide, FAD; flavin mononucleotide, FMN; free riboflavin, FR; 2-heptyl-4-OH-quinoline-N-oxide, HOQNO; *p*-chloromercuribenzoate, PCMB.

* A part of this study was reported in the 11th Symposium on Enzyme Chemistry held at Kyoto, Japan and in the Symposium on Haematin Enzymes held at Canberra in 1959[1].

INTRODUCTION

Recent studies on the enzymic mechanism of microbial nitrate reduction have shown that the reduction does not have a single mechanism and is of no common metabolic significance. The multifunctional nature of nitrate reduction can be now understood by classifying it into two types[2-4].

The first type, "nitrate respiration type", has been found in the group of facultative anaerobes such as *E. coli*. *E. coli* NaR which has been studied in details in this laboratory was, one decade ago, suggested by SATO *et al.*[5] to be an iron enzyme. Then, the enzyme from aerobically grown *E. coli* cells was confirmed by TANIGUCHI *et al.*[6] and IIDA *et al.*[7] to be a terminal enzyme in a particulate electron-transferring system with cyt. b_1 functioning as the indispensable electron carrier. The particulate NaR together with cyt. b_1 has been solubilized by deoxycholate or iso-butanol treatment[7] and the properties of the soluble enzyme systems have been also described[7-9]. Recently, highly active NaR of anaerobically grown *E. coli* cells solubilized by steapsin or chymotrypsin digestion and partially purified was tentatively suggested by TANIGUCHI *et al.*[9] as a metalloflavoprotein. However, any definite conclusions on the chemical nature of the enzyme have never been drawn.

On the other hand, the second type, "nitrate assimilation type", has been found in the group of aerobes such as *Neurospora*. *Neurospora* enzyme, extensively studied by NASON's group in the McCollum–Pratt Institute, has been proved to be a soluble molybdoflavoprotein[10] which does not include any participation of *Neurospora* cytochromes[3].

The present paper describes the solubilization and purification of NaR to a homogeneous state from a particulate electron-transferring system of nitrate-respiring *E. coli* cells together with the physical and chemical characterization of the homogeneous enzyme as not a metalloflavoprotein but as a metalloprotein.

MATERIALS AND METHODS

Micro-organism

E. coli, Yamagutchi strain, was anaerobically grown in 70 l of a medium of the following composition in a 100 l stainless steel tank at 30° with occasional N_2 gas bubbling: 1 % polypeptone (Wako Chemicals), and meat extract paste (Kyokuto Chemicals), 0.1 % KNO_3, casamino acid (Difco Chemicals), and yeast extract powder (Wako Chemicals), 0.2 % K_2HPO_4 and 2.0 % glucose, pH 7.2. The liquor of young cells preliminary grown under the same conditions was inoculated to produce the concentration of 5 % (v/v). As soon as the logarithmic growth phase was accomplished (about 5.5–7.0 h after the inoculation), the liquor was immediately cooled on ice and any solid formed was separated and washed with distilled water; nitrite was removed by a Sharples ultracentrifuge in the cold. The cell paste obtained can be stored in a frozen state at —15° for about 2 weeks without any noticeable loss of MVH-NaR activity.

Cell disruption and preparation of particulate NaR

All the procedures were carried out below 4° unless otherwise described. The frozen cells were ground in a mortar for 30 min with twice their weight of alumina powder (Wako No. 800) and for another 10 min with five times their weight of cold 0.1 *M* phosphate buffer, pH 7.1 and then centrifuged at 2000 × *g* for 20 min to obtain the cell-free extract. By centrifuging the cell-free extract at 15,000 × *g* for 30 min, the particulate preparation was obtained as a precipitate; this was washed twice with cold distilled water. The particulate preparation of thick paste can be stored in a frozen state at −15° for about 2 weeks without any noticeable loss of MVH-NaR activity.

Other materials

Calcium phosphate gel was prepared according to the method of KEILIN *et al.*[11] and was aged before use for at least a week. The gel was adjusted to contain about 30 mg dry weight per ml of gel. DPNH and TPNH were prepared by the enzymic reduction of the respective nucleotides of 70% or more purity by the methods of PULLMAN *et al.*[12] and by EVANS *et al.*[13], respectively. Authentic samples of FAD and FMN were kindly donated by Dr. J. OKUDA. MV and BV were purchased from British Drug Houses, Ltd., and were used after recrystallization. HOQNO was kindly donated by Dr. R. SATO and used according to the method described by LIGHTBOWN *et al.*[14]. Mammalian cyt. *c* was purchased from L. Light and Co. Ltd.

Solubilization and purification of the particulate NaR preparation

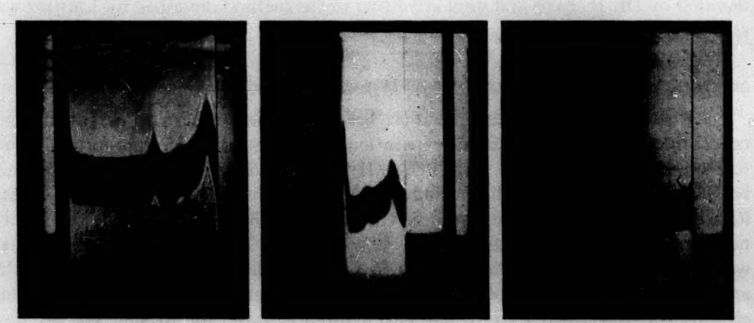
All procedures were carried out at below 4° unless otherwise described. The concentration of the particulate preparation was adjusted to an appropriate value (0.2–0.6 mgN/ml) in 5 m*M* Na_2HPO_4 (pH 8.3) containing 0.1 m*M* of KNO_3 and heated at 60° for 5 min. The heated suspension was immediately cooled to 4° by ice-water and kept for about 15–20 h. The soluble NaR was obtained as the supernatant after Sharples centrifugation at 50,000 × *g* for 10 min. To this supernatant, was added 1 *M* acetate buffer, pH 5.0 to produce a final concentration of 0.02 *M* and calcium phosphate gel of 70–150 mg dry weight per 100 ml of supernatant; the solution was then stirred for 10 min and allowed to stand for 10 min. The gel was collected and twice washed with distilled water and once with 2–10 m*M* phosphate buffer, pH 7.1. A large proportion of the adsorbed enzyme could be eluted from the gel by a phosphate buffer of 0.1 *M*, pH 7.1 containing 0.1 m*M* glutathione as an enzyme stabilizer. The combined eluates (about 80 ml from 100 g cell paste) were fractionated with $(NH_4)_2SO_4$ with standing for 20 min after the slow addition of the solid salt to obtain the fraction at between about 38–53% saturation. The precipitate was dissolved with about half the volume of the same medium and the fraction between about 30–40% saturation was again obtained in the same manner. The resulting precipitate was dissolved in 0.05 *M* phosphate buffer pH 7.1 containing 0.1 m*M* of glutathione of volume one quarter that of the original eluate and was subjected to ultracentrifugation in a Spinco Model L at 110,000 × *g* for 3–4 h. Owing to the large molecular weight of NaR described later, the active material was precipitated and washed three times with the same medium in order to remove the inactive lighter components. Typical results of the solubilization and purification together with the ultracentrifugal patterns of the intermediate and final fractions are summarized in Table I.

In steps 3, 4 and 5 as designated in Table I, preliminary small scale tests must always be conducted to determine the most favorable conditions for each purification. The final preparation can be stored for one week in the same phosphate buffer containing glutathione in a frozen state at —15° with less than 30–40 % loss of MVH-NaR activity.

TABLE I

SOLUBILIZATION AND PURIFICATION OF *E. coli* NITRATE REDUCTASE

Step No.	*Fraction*	*Total N (mg)*	*Units ($\times 10^{-4}$)*	*Specific activity ($\times 10^{-3}$)*
1	Cell paste	(270 g wet weight)		0.18
2	Particulate preparation	2500	135	0.54
3	Supernatant solubilized after heat treatment	580	100	1.72
4	Calcium phosphate gel eluate	227	90	3.95
5	$(NH_4)_2SO_4$ 1st ppt. (38–53 % sat.)	55.2	80	14.5
6	$(NH_4)_2SO_4$ 2nd ppt. (30–40 % sat.)	11.2	50	56.2
7	Spinco L 1st pellet	3.04	40	132
9	Spinco L 3rd pellet	1.50	28	186



Step 5 Step 7 Step 9

Sedimentation patterns of nitrate reductase preparations in phosphate buffer, pH 7.1, ionic strength, 0.1. The direction of sedimentation is from right to left and the photographs were taken 24 min after the rotor attained full speed (59,780 r.p.m.). In these experiments, average rotor temperature was 13.5°, protein concentration approximately 0.5 %, and bar angle 55°. The activity was always shown to be associated with the fastest peak in each pattern.

Solubilization of the particulate NaR preparation by dodecylsulfate treatment

To the particulate preparation suspended in 0.05 *M* phosphate buffer, pH 7.1 in 3–5 mg N/ml concentration, was added a 10 % solution of dodecylsulfate to give a final concentration of 0.5–1.0 %. After standing for 2 h at 30°, a reddish brown supernatant was obtained after centrifugation at 20,000 × *g* for 40 min and this was used as the dodecylsulfate solubilized preparation.

Solubilization of the particulate NaR preparation by steapsin or chymotrypsin treatment

The method of solubilization by means of steapsin (Nutritional Biochemical Corp. from pig pancreas) was previously described by the authors[9]. Using the same conditions as for the steapsin method, chymotrypsin (Nutritional Biochemical Corp.)

could be applied in place of steapsin. Pepsin, trypsin, lipase (from *Sclerotinia libertiana*), and crystalline proteases (from *B. subtilis* and *Streptomyces griseus*) were shown to be without effect under the above conditions.

Electrophoretic, ultracentrifugal and spectrophotometric analyses

Criteria of purity for the enzyme preparations were examined with a Spinco Model H electrophoretic apparatus and a Spinco Model E ultracentrifuge. Spectrophotometric analysis was carried out with a Beckman spectrophotometer DK-2 or a Hitachi electrospectrophotometer Model ERB-U.

Determination of enzyme-bound Mo by spectrographic analysis

To determine the Mo content in the homogeneous preparation, an enzyme sample and, as a blank test substance, the almost inactive protein which could be separated at step 7 in Table I as an ultracentrifugal supernatant were simultaneously dialyzed for 20–50 h at 4° in the same bottle against deionized distilled water with stirring and one exchange of the outer solution after several hours from the start of the dialysis. After dialysis, equal volumes of aliquots of the enzyme sample, inactive protein and the outer solution were placed in clean glass tubes and lyophilized. After drying the lyophilized samples for 3 h in an Abderhalden apparatus at 100°, the weights of the three samples were determined. Usually 3–7 mg of the protein samples were oxidatively decomposed by 1.0 ml of fuming nitric acid and 0.5 ml of 30% hydrogen peroxide. After adding the spectroscopically pure graphite powder in approximately equal amounts to those of the protein samples, the mixtures were evaporated to dryness under an infra-red lamp at 110° for 4 h. Definite amounts of the dried materials were then well mixed in an agate mortar with approximately equal amounts of spectroscopically pure sodium chloride which was found to enhance the emission lines, and then the mixtures were subjected to emission spectrography using the cathode layer method. The Mo content actually found in the enzyme sample should be corrected for the minute contents of Mo (about 10–15% of that of the enzyme sample) found in the inactive protein and the outer solution (in both samples the contents were approximately equal) probably resulting from the phosphate salt reagent used as the medium in the enzyme preparation. The determination was carried out by Drs. C. IIDA AND K. YAMASAKI in the Laboratory of Inorganic Chemistry of our Department. For the details of the procedures employed, their papers[15, 16] should be consulted.

Determination of enzyme-bound total iron

As already described in the procedure for Mo determination, 0.5 ml of 1 *N* HCl was added to the appropriate volume of the dialyzed enzyme sample and inactive protein or the outer solution employed as the blank material, and decomposed by heating. After the addition of 2.0 ml of 2 *M* acetate buffer, pH 4.0, 5.0 ml of 10% aqueous solution of NH_2OH–HCl and a suitable amount of ammoniacal aqueous solution (1:3) to maintain the final pH at 4.0 correctly, the mixture was allowed to stand for 5 min at room temperature to completely reduce the ferric iron. After developing the green color by the addition of *o*-nitrosoresorcinol monomethyl ether reagent described by TORII[17], the colored solution was washed three times with CCl_4 and centrifuged to remove the turbidity due to any remaining CCl_4. Then, the total iron was determined colorimetrically (at a wave length of 700 mμ) for the clear supernatant.

Determination of enzyme-bound flavins

The determination was carried out fluorometrically by the lumiflavin method[18] and D-amino acid oxidase assay[19]. In the latter assay, the homogeneous preparation which was boiled for 10 min in a neutral pH condition was treated.

Enzyme assay

(a) *MVH method*: This assay was carried out on a mixture containing 0.05 μmole of MV, 50 μmoles of KNO_3, 250 μmoles of phosphate buffer, pH 7.1 and the enzyme solution with a total volume of 0.5 ml in an open tube at 30°. After 5 min preincubation, the reaction was started by the addition of small amounts (less than 1 mg) of dithionite. After the reaction had proceeded for 5 min, the tube was immediately shaken to oxidize the remaining MVH by the air. The amount of nitrite formed was colorimetrically determined by Griess-Ilosvay reagent. This activity is referred to MVH–NaR in this paper. In testing the other donors, MVH was replaced by others. In the assay of the activities of the particulate preparation, the reaction was carried out in a Thunberg tube in order to restrain the particulate oxidase activity.

(b) *Spectrophotometric method*: Sometimes, the activity was spectrophotometrically assayed utilizing a reduced dye as the electron donor by measuring the change in the characteristic absorption of each reduced dye (*i.e.*, in the case of MVH, by measuring the decrease of the optical density at 590 mμ and using the corresponding molecular extinction coefficient of MVH which has been determined to be $7.65 \cdot 10^6$ cm^2/mol at pH 7.1) using a Thunberg tube-type cuvette. In these cases, a stoichiometrical relationship between the amounts of the reoxidized dye and the nitrite formed was established.

Definition of NaR units and the specific activity

One unit of MVH–NaR was defined as the amount of the enzyme which produced 1 μmole of nitrite in 1 h under the above conditions. The specific activity was defined as units per mg Kjeldahl nitrogen.

Other assay methods

The assay method with Mb and the expression of the units and the specific activity for FDH and other dehydrogenases was as previously described[7]. The protoheme content in the particulate preparation was determined by the method of Moss[20].

RESULTS

Nitrate-respiring E. coli *cells*

The multi-functional character of the nitrate reduction of *E. coli* has already been reported by TANIGUCHI *et al.*[2] who demonstrated that this microbe can perform simultaneously two different types of nitrate metabolism: nitrate respiration (anaerobiosis) and apparent nitrate assimilation (aerobiosis).

The former activity can be shown by measuring the anaerobically reduced amount of nitrate in the presence of a suitable electron donor such as formate. The anaerobic reduction of nitrate cannot proceed further than the nitrite stage and is profoundly retarded by low oxygen pressure, reflecting the character of this nitrate respiration[2].

On the other hand, the latter activity can be detected by measuring the aerobically reduced amount of nitrate in the presence of a suitable respiratory substrate such as formate[21]. Under the aerobic conditions, accompanying the simultaneous oxidation of formate, nitrate can be reduced to nitrite and to ammonia which is also rapidly metabolized suggesting an apparently assimilative character of the aerobic reduction[2, 21].

As shown in Table II, *E. coli* cells grown aerobically in the standard medium with shaking possess much higher activity of aerobic reduction than that of anaerobic reduction. In the anaerobically grown cells, on the contrary, much lowered activity of aerobic reduction and about 20-fold enhanced activity of anaerobic reduction can be found. The addition of nitrate to the medium which supports anaerobic growth was found to stimulate not only the bacterial growth and formate–NaR activity but also to enhance the cyt. b_1 content of the cells. Thus, *E. coli* cells anaerobically grown by apparently respiring nitrate and used in the present study were shown to possess an active mechanism for nitrate respiration.

TABLE II

NITRATE-REDUCING ACTIVITIES OF *E. coli* CELLS

Reaction mixture: KNO_3, 100 μmoles; formate, 1 μmole; phosphate buffer pH 7.1, 0.08 *M*; cell suspension. Total volume, 3.0 ml at 30°. Figures represent the reduced NO_3^- (μmoles) per h and mg N of the cells.

Reaction condition	*Growth condition*	
	Aerobic	*Anaerobic*
Aerobic* ($\longrightarrow NH_3 \dashrightarrow$)	30–50	0–5
Anaerobic** ($\longrightarrow NO_2^-$)	10–20	200–500

* The reaction was carried out in a Warburg vessel with vigorous shaking under air and the nitrate reduced was measured according to the procedure previously described[2, 21].

** The reaction was carried out in a Thunberg tube under anaerobic conditions and the nitrite produced was measured according to the procedure previously described[7].

Particulate NaR system and the solubilized NaR systems

Examination of localization patterns of a NaR system by centrifugal fractionation of the cell-free extract revealed that about 70 % of the units of MVH–NaR, formate–NaR and protoheme contents together with about twice* the number of FDH units which were initially found in the cell-free extract were concentrated in the particulate preparation. The specific activities of the MVH–NaR and FDH of the particulate preparation have the values of 200–1000 and 300–1000, respectively. The present localization patterns are essentially the same as those previously reported in the case of aerobically grown *E. coli* cells[2,7] indicating that these enzymes are bound to cellular structures.

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* This suggests the presence in the cell-free extract of some uncharacterized factor inhibiting FDH activity.

For the enzymic reduction of nitrate by the particulate preparation, as shown in Table III, natural electron donors such as $FMNH_2$, formate and DPNH (effective in this order) but not TPNH could be utilized. Among the artificial donors, MVH and BVH, both one-electron donors with very low redox potentials, were the most effective.

TABLE III

COMPARISON OF ELECTRON DONORS FOR PARTICULATE *E. coli* NITRATE REDUCTASE ACTIVITY

Electron donors except formate, DPNH and TPNH were reduced by dithionite as described in MVH method. Concentrations of these electron donors were all in sufficiently saturating levels for the particulate NaR activity.

Electron donor (2 mM in final concentration)	*Units (relative value)*
Methylviologen-H	100
Benzylviologen-H	100
Phenosafranine-H_2	58
Pyocyanine-H	55
Methylene blue-H_2	5.3
Phenazine methosulfate-H	2.7
FMN-H_2*	31
FAD-H_2*	29
Formate**	16
DPNH*	6.8
TPNH*	0–2

* 0.2 m*M*.
** 50 m*M*.

The difference spectrum (reduced minus oxidized) of the particulate preparation of which the reduced state was anaerobically maintained by the FDH activity is shown in Fig. 1.

The peaks of the difference spectrum at 432, 532, and 560 mμ clearly indicate the abundant presence of cyt. b_1 in the particulate preparation. As seen from the

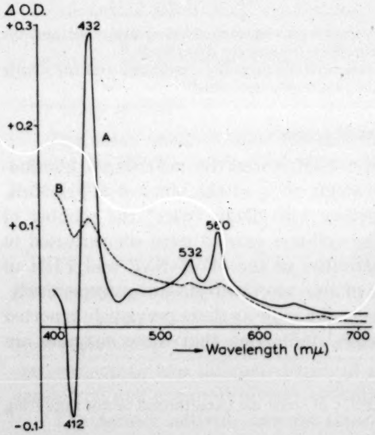

Fig. 1. Difference spectra of the *E. coli* particulate preparation. When the particulate preparation of 1.8 mg N was anaerobically incubated in the presence of 50 μmoles of phosphate buffer, pH 7.1 and sodium formate in a Thunberg tube-type cuvette for 15 min, the difference spectrum (reduced minus oxidized) was observed (Curve A). The spectrum obtained by reducing with formate was very similar to that reduced with dithionite except that partial reduction (about 80%) was observed with formate. After adding 10 μmoles of solid KNO_3 from the side tube into the main tube, the difference spectrum (Curve A) changed to Curve B within 1–2 min.

same figure, cyt. b_1 enzymically reduced by formate was instantaneously re-oxidized by the addition of nitrate together with the simultaneous production of nitrite.

As shown in Table IV, formate–NaR activity is strongly inhibited by HOQNO, the specific inhibitor for cyt. b_1. The same extent of inhibition of DPNH–NaR activity by quinoline oxide was also found whereas no inhibition of the MVH–NaR activity by the compound could be confirmed. These observations led the authors to the conclusion that cyt. b_1 participated in the reaction as an indispensable intermediary carrier for the formate– and DPNH–NaR but not for the MVH–NaR. Table IV also shows that formate–NaR activity differs remarkably from MVH–NaR activity in its sensitivity towards amytal, dicumarol (never reversed by menadione) and PCMB suggesting the participation in the former system of flavoprotein, a dicumarol-sensitive site and an –SH group, respectively. Among the redox dyes tested, only phenazine methosulfate caused noticeable activation probably owing to its functioning as an effective intermediary carrier in place of cyt. b_1. Cyanide and azide at 0.1 m*M* final concentrations resulted in an appreciable inhibition (65 and 80 %, respectively) of both formate– and MVH–NaR activities, indicating a heavy metal component probably in the common terminal enzyme of the particulate system. Various chelating reagents tested* caused no remarkable inhibition.

TABLE IV

EFFECT OF INHIBITORS AND ACTIVATORS ON THE PARTICULATE *E. coli* NITRATE REDUCTASE ACTIVITY

Compound	*Final concentration (M)*	*Inhibition (%)*	
		from formate	*from MVH*
Amytal	$2 \cdot 10^{-3}$	75	20*
Menadione**	$5 \cdot 10^{-3}$	40*	0
Dicumarol	$3 \cdot 10^{-5}$	70	25
Dicumarol + Menadione	$(3 \cdot 10^{-5}) + (5 \cdot 10^{-3})$	70	—
HOQNO	10^{-5}	85	0
PCMB	$5 \cdot 10^{-4}$	60	20
Phenazine methosulfate	$3.3 \cdot 10^{-4}$	160*	5

* Activation per cent.

** Ethanol solution was used with the appropriate control.

Michaelis constants (K_m) of the particulate formate–NaR for nitrate and formate were estimated to be $1.3 \cdot 10^{-4}$ *M* and $7.0 \cdot 10^{-4}$ *M*, respectively.

Regardless of the kind of buffers tested (glycyl–glycine, Tris and phosphate), the pH optimum for the particulate formate–NaR activity was 7.0–7.2 and the activity was not specifically affected.

The particulate preparation was found to be devoid of not only dehydrogenase activity (Mb assay) for lactate, succinate, β-OH-butyrate and glucose, but hydrogenase (H_2 production from MVH), DPNH and TPNH oxidase activities. Besides the very strong activity of FDH, only a weak activity of formate oxidase was found.

Summarizing the results described above, the particulate electron-transferring system to the nitrate of *E. coli* of nitrate respiration type can be illustrated by the following scheme.

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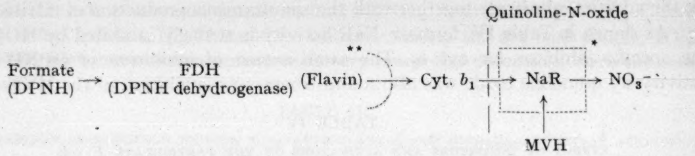
* Chelators tested were the same as those described in the footnote on p. 277.

PARTICULATE *E. coli* NITRATE REDUCTASE SYSTEM



* Only the terminal moiety is solubilized by either alkaline cold incubation, or steapsin or chymotrypsin digestion after heat treatment.

** This functional connection can be split by dodecylsulfate treatment which simultaneously solubilizes all the entities FDH, cyt. b_1 and NaR bound in the particulate preparation.

The recovery of the activities of particulate enzymes in the solubilized preparations which can be brought about by three different procedures—alkaline cold incubation after heat treatment, steapsin or chymotrypsin treatment and dodecylsulfate treatment is summarized in Table V. The former two treatments can specifically solubilize only MVH–NaR activity. On the other hand, dodecylsulfate treatment can solubilize a large proportion of the FDH activity and cyt. b_1 whereas formate– and DPNH–NaR activities have never been detected in the solubilized preparation. However, as in the case of the original particulate preparation, it was found that a reduced form (with dithionite) of cyt. b_1, present in the dodecylsulfate-solubilized preparation, was quite an effective electron donor for nitrate reduction by the preparation. As shown in the previous scheme, it appears that a factor functionally connecting DPNH and formate with cyt. b_1 was removed by the dodecylsulfate treatment while alkaline incubation more specifically cleaved only the terminal entity, MVH–NaR. A similar mode of cleavage was found also in the case of solubilization by means of steapsin or chymotrypsin. The highly active preparations were purified chromatographically (Dowex-2) after solubilization by hydrolyzing enzymes[9] and were found to exhibit several diffuse peaks with a slow rate of

TABLE V

RECOVERY OF ACTIVITIES OF PARTICULATE ENZYMES IN THEIR SOLUBILIZED PREPARATIONS

Fraction	*Relative units (particulate preparation taken as 100)*				*Relative content of cyt. b_1***
	MVH → NaR	*FDH (→ Mb)*	*formate → NaR*	*DPNH → NaR*	
Particulate preparation	100	100	100	100	+++
Solubilized supernatant prepared by:					
Alkaline cold incubation after heat treatment	70*	0	0	0	±
Steapsin or chymotrypsin digestion after heat treatment	85*	0	0	0	±
Dodecylsulfate treatment	95*	75*	0	0	++

* These activities were found to remain in the high speed supernatant (110,000 × g for 1 h) of each solubilized preparation.

** Intensity of α-band (560–565 mμ) of cyt. b_1 reduced with dithionite was spectroscopically observed.

sedimentation in the patterns from the ultracentrifugal analysis which indicated heterogeneity probably caused by enzymic splitting of the NaR molecule*. For the purpose of enzyme purification the milder procedure of solubilization by cold alkaline incubation after heat treatment was advantageously employed. The effect of dodecylsulfate treatment is similar to that of isobutanol treatment employed for the particulate preparation from aerobically grown *E. coli* cells[7].

Physical and chemical characterizations of homogeneous NaR

Molecular constants and turnover number of NaR: The sedimentation and electrophoretic patterns of the final preparation are shown in Table I and Fig. 2, respectively. A single component with a symmetrical pattern associated with the brown–yellow color of the enzyme was found in each analysis. The electrophoretic mobility of the enzyme at pH 7.1 was estimated under the conditions stated in Fig. 2 to be —9.55 · 10^{-5} cm^2 volt^{-1} sec^{-1}.

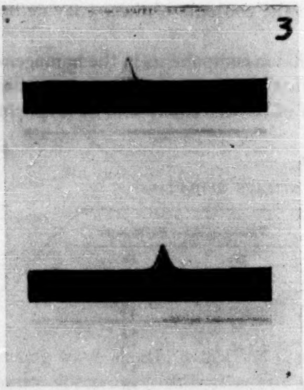


Fig. 2. Electrophoretic patterns of nitrate reductase (specific activity, 180,000) in phosphate buffer, pH 7.1, ionic strength, 0.1. In this experiment, the time of electrophoresis was 120 min, protein concentration, 0.5 %, current, 2.5 mA and temperature, 2.2°. Upper diagram: ascending pattern. Lower diagram: descending pattern.

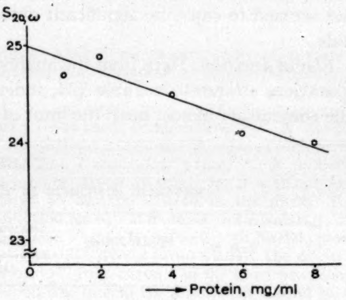


Fig. 3. Plot of $S_{20,w}$ of *E. coli* nitrate reductase against protein concentration.

The measurement of the molecular weight of the enzyme was carried out by Dr. N. UI of Gunma University. From the data presented in Fig. 3, the sedimentation coefficient ($S_{20,w}$) of nitrate reductase was estimated by extrapolation to zero concentration to be 25.0 Svedberg units. The measurement of the diffusion constant in the same buffer and at 0.4 % protein concentration showed that the value of $D_{20,w}$ was 2.27 · 10^{-7} cm^2 sec^{-1}. Assuming the partial specific volume of the protein to be 0.75 ml g^{-1}, the molecular weight of nitrate reductase was calculated to be a million. By assuming that the nitrate reductase molecule is a prolate ellipsoid without hydration, the frictional ratio (f/f_0) estimated to be 1.38 gives an axial ratio of 7–8 suggesting a considerable deviation of the molecular shape from a spherical one. On the basis of this established molecular weight, the specific activity of MVH–NaR of the final preparation of $13\text{–}22 \cdot 10^4$ gives the turnover number of the nitrate reductase as $5.2\text{–}8.8 \cdot 10^3$ moles/sec.

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* For the experimental evidence, see the legend for Table VII.

Enzyme-bound metals: The results of spectrographic analysis revealed the characteristic presence of Mo and Fe in the homogeneous enzyme samples. Besides these metals, small but significant amounts of Mg and trace amounts of Mn were detected. However, the presence of almost equal amounts of these metals were confirmed in the blank materials.

As shown in Table VI, on the basis of a molecular weight of a million and the dry weight of the enzyme protein, the Mo content determined indicates that the nitrate reductase has one atom of Mo per molecule. On the other hand, the Fe content determined colorimetrically indicates the presence of about 40 non-heme Fe atoms per molecule. Prolonged dialysis of the enzyme protein against deionized distilled water seemed to cause no significant change in the contents of these enzyme-bound metals.

Flavin detection: Data from the analysis of flavin components in the homogeneous preparation, as shown in Table VII, indicate that the detectable flavin components in the enzyme are almost near the limit of error of the determination. It is concluded

TABLE VI

METALLIC CONTENTS IN *E. coli* NITRATE REDUCTASE

Expt. No.	*Specific activity* (× 10^{-4})	*Dialysis time against deionized water* (*h*)	*Number of atoms per molecule*	
			Mo	*Fe*
1	16	20	1.2–0.8*	45
2	18	15	—	43
		50	1.1–0.7**	36
3	18	36	—	42
4	14	30	—	25

* Determined by the internal standard method with Pd and V[15,16].
** Determined by the visual method[15,16].

TABLE VII

FLAVIN DETECTION

Expt. No.	*Specific activity of nitrate reductase* (× 10^{-4})	*Detected as free riboflavin* (*μg/g dry weight*)	*Number of flavin molecules per molecule of nitrate reductase*
1	22	2.8	0.009
2	22*	0.0	0.000
3	18	0.8	0.002
4	15	0.2	0.0005
5	20	—	0.02**

* The enzyme protein used in expt. 1 was digested by chymotrypsin of weight 8% that of NaR at pH 8.15 and 37° for 3 h. By ultracentrifugal analysis the digested mixture was shown to consist of almost a single component having a smaller value of sedimentation coefficient in spite of no remarkable loss of activity during the digestion. For this digested mixture, fluorometric assay was carried out.
** This was determined by D-amino acid oxidase assay.

therefore, that the enzyme-bound flavin component is, if any, a trace and not significant in its amount which indicates that the present final product is a metalloprotein but not a metalloflavoprotein. From the authors' previous data including the absorption spectra of the partially purified preparation, NaR was temporarily regarded as a metalloflavoprotein[9]. In fact, the partially purified preparation (*i.e.*, preparation at step 5) showed a spectrum similar to that previously presented[9]. However, large portions of the contaminated inactive flavoproteins were shown to be effectively eliminated in the initial $(NH_4)_2SO_4$ precipitate in steps 5–6 and also in the ultracentrifugal supernatant in steps 7–9.

Absorption spectra: As seen from Fig. 4, the homogeneous preparation had no specific absorption peak except that of protein in the vicinity of 277 mμ and the absorbance decreased gradually over the entire near u.v. and visible region with increasing wave length, indicating none of the characteristics of a heme enzyme. The difference spectrum (oxidized minus reduced) showed a broad peak at 445–450 mμ which instantly disappeared under anaerobic conditions on the addition of nitrate with the simultaneous production of nitrite. However, the oxidized spectrum could not be transformed by means of DPNH to a reduced one such as that formed with $NaBH_4$. The reduced spectrum was not affected by darkness and anaerobic incubation with 1 atm. of CO. A deproteinization procedure for the homogeneous preparation which consisted of treatment with cold trichloroacetic acid (5 % of final concentration), yielded a completely colorless supernatant and a yellow precipitate of the denatured enzyme protein. Though the nature of these spectra cannot yet be fully understood, the brown–yellow color may presumably be due to combination between the enzyme protein and heavy metals.

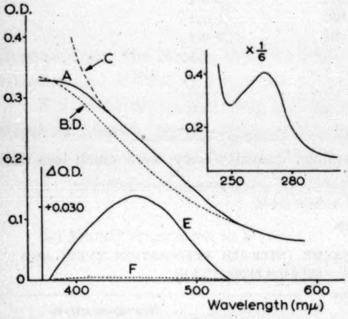

Fig. 4. Absorption spectra of *E. coli* nitrate reductase. The homogeneous enzyme preparation of specific activity, 150,000 and of 1.9 mg protein/ml concentration in 0.02 *M* phosphate buffer, pH 7.1 was used. Temperature, 25°: A, oxidized form (without additions); B, reduced with $NaBH_4$ (less than 1 mg) (after 5 min); C, A + DPNH (3 μmoles) (after 30 min); D, B + CO (1 atm.) (after 30 min incubation in the dark); E, A (oxidized form) – B (reduced with $NaBH_4$); F, A (oxidized form) – B (reduced with $NaBH_4$ plus 10 μmoles solid KNO_3) (after 5 min). The deviation of curve C from curve A in the short wave length region was due to the absorption caused by the DPNH added.

Other properties: Table VIII shows that MVH and BVH are still the most effective electron donors for the activity of the homogeneous preparation in view of their values of both V_{max} and K_m. There seems to be no essential difference in the reactivity with the electron donors listed in Table VIII from those exhibited by the particulate enzyme (Table III) except that the reactivities for reduced flavin derivatives exhibited by the purified enzyme are relatively much reduced. Ferricyanide, $FeSO_4$, ascorbate, reduced mammalian cyt. *c*, reduced cyt. c_3*, hypoxanthine and DPNH were all inactive as electron donors for nitrate reduction. No detectable activity of MVH–, DPNH– and TPNH–cyt. *c* (mammalian) reductase, aldehyde or xanthine dehydrogenase (from acetaldehyde or hypoxanthine to indophenol dye), even in the presence of externally added FAD, was confirmed in the homogeneous preparation. Remarkable sensitivities for cyanide and azide but not for various chelators tested* were seen for the MVH–NaR activity representing essentially

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* The purified sample of cyt. c_3 was kindly donated by Dr. M. Ishimoto.

TABLE VIII

COMPARISON OF ELECTRON DONORS FOR *E. coli* NITRATE REDUCTASE

The spectrophotometric assay method was employed.

Electron donor (reduced with dithionite)	*Vmax. (relative value)*	*K_m for the donor ($\times 10^{-5}$ M)*
Methylviologen-H	100	$>$0.1*
Benzylviologen-H	100	$>$0.1*
Phenosafranine-H_2	84	$>$0.1
Methylene blue-H_2	2.8	4.5
FAD-H_2	1.2	1.3 — 2.0
FMN-H_2	6.0	2.6
FR-H_2	3.2	1.6

* These figures, though they could not be determined quantitatively, were much less than those for phenosafranine-H_2.

TABLE IX

COMPARISON BETWEEN PROPERTIES OF *E. coli* ENZYME (NITRATE RESPIRATION TYPE) AND *Neurospora* ENZYME (NITRATE ASSIMILATION TYPE)

	E. coli enzyme	*Neurospora enzyme*
Enzyme-bound components:		
Mo	1 atom /molecule	Present
Fe	~40 atoms/molecule	Not reported
flavin	Not detected	FAD
Preferable electron donor	Reduced methylviologen	Reduced indophenol
Physiologically required electron donor	Reduced cyt. b_1	TPNH
K_m for NO^-_3	$5.1 \cdot 10^{-4}$ M (from reduced methylviologen)	$1.4 \cdot 10^{-3}$ M (from TPNH)
Cyt. *c* reductase activity	Not detected	Actively associated
Cytochrome participation	Cyt. b_1	None
Original form	Particulate	Soluble
Physiological function	Nitrate respiration	Nitrate assimilation

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* 8-OH-quinoline, *o*-phenanthroline, α,α′-dipyridyl, diethyl dithiocarbamate (these in 1 m*M* final concentration), *o*-nitrosoresorcinol monomethyl ether (0.24 m*M*), thioglycolate (10 m*M*) and allyl thiourea (0.5 m*M*) produced only about 10–30 % inhibition. Neither NaF, ethylenediamine tetraacetic acid (these in 10 m*M*), salycylaldoxime (1 m*M*) nor dithizon (80 μ*M*) exhibited any inhibitory effect.

similar sensitivities to those in the case of particulate MVH–NaR activity. Moreover, the same sensitivities as described above were also found for $FADH_2$–NaR activity. No significant sensitivity for CO in the dark was found for not only the activities of MVH- and $FADH_2$–NaR of the homogeneous preparation but also those of formate–, DPNH–, and MVH–NaR of the particulate preparation.

The optimum pH for MVH–NaR activity was 7.1 in phosphate buffer, which was found to be a more effective buffer for restoring MVH–NaR activity than Tris, citrate and pyrophosphate buffers. The Michaelis constant (K_m) for the MVH–NaR of the homogeneous preparation for nitrate was estimated to be $5.1 \cdot 10^{-4}\,M$. Heat treatment at 60° for 5 min resulted in no inactivation while treatment at 70° caused 70% inactivation.

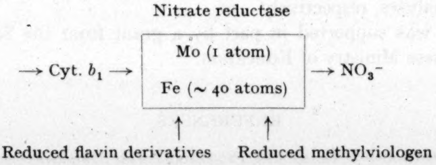
DISCUSSION

Summarizing the results described above, a comparison of properties between *E. coli* enzyme and *Neurospora* enzyme is presented in Table IX.

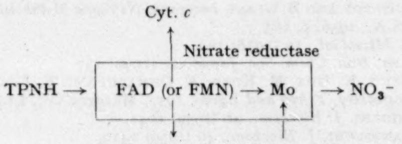
The electron-transferring sequences of these two NaR systems are also schematized as follows. In the light of the general functioning of Mo recently established in the NaR of *Neurospora*[10, 22] and higher plants[23] or in the action of xanthine dehydrogenase from milk and liver[24–26], Mo found in the enzyme protein seems to take part in the terminal electron transfer of the nitrate reduction. On the other hand, the presence of bound iron atoms has not been reported for the *Neurospora* enzyme. The function and significance of the iron atoms still remain to be elucidated. It is of some interest, however, that the large molecule, succinate dehydrogenase, a flavoprotein of molecular weight of about several millions, was recently isolated from *M. lactilyticus* and was shown to bind multiple non-heme iron atoms of which the

ELECTRON-TRANSFERRING SEQUENCES OF *E. coli* NITRATE REDUCTASE AND OF *Neurospora* NITRATE REDUCTASE

(a) Nitrate respiration in *E. coli*:



(b) Nitrate assimilation in *Neurospora*[10]:



function is also not yet clarified[28]. The other most noticeable difference from the *Neurospora* enzyme is that the *E. coli* enzyme has no significant level of bound flavin component and neither DPNH nor TPNH, but possibly reduced cyt. b_1 can specifically function as a physiological and direct electron donor. *E. coli* enzyme has been purified by the milder procedure in which, generally speaking, the possibility that a flavin moiety would be released from an enzyme or would be decomposed seems improbable.

The present enzyme cleaved from the terminal moiety of the particulate system of *E. coli* grown anaerobically in the presence of nitrate may be regarded as an anaerobic variant of the terminal oxidases and the functional system of nitrate respiration *in vivo*.

Evidence for the functioning of Mo by valency change in enzymic reactions as well as data indicative of the nature of the bound iron atoms and the acceptor specificity of the enzyme will be presented in a second paper[29].

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Professor F. Egami for his valuable and encouraging suggestions, and to Mr. K. Ohmachi for his kind collaboration and discussions throughout this work. The authors are deeply indebted also to Drs. N. Ui and C. Iida for their co-operation. Thanks are also due to Drs. J. Okuda, K. Takata and Mr. S. Tsukamoto of Nagoya University for making facilities available for carrying out the fluorometric flavin assay, for carrying out the electrophoretic and ultracentrifugal analyses, respectively.

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REFERENCES

[1] F. Egami, M. Ishimoto and S. Taniguchi, *Proceedings of the Symposium on Haematin Enzymes*, Canberra, 1959, in the press.
[2] S. Taniguchi, R. Sato and F. Egami, in W. D. McElroy and B. Glass, *Inorganic Nitrogen Metabolism*, The Johns Hopkins Press, Baltimore, U.S.A., 1956, p. 87.
[3] R. Sato, in W. D. McElroy and B. Glass, *Inorganic Nitrogen Metabolism*, The Johns Hopkins Press, Baltimore, U.S.A., 1956, p. 163.
[4] A. Nason, *Ann. Rev. Microbiol.*, 12 (1958) 203.
[5] R. Sato and F. Egami, *Bull. Chem. Soc. Japan*, 22 (1949) 137.
[6] S. Taniguchi, A. Asano, K. Iida, M. Kono, K. Ohmachi and F. Egami, *International Symposium on Enzyme Chemistry, Tokyo and Kyoto, 1957*, Maruzen Co., Ltd., Tokyo, 1958, p. 238.
[7] K. Iida and S. Taniguchi, *J. Biochem.*, 46 (1959) 1041.
[8] E. Itagaki and S. Taniguchi, *J. Biochem.*, 46 (1959) 1419.
[9] S. Taniguchi and E. Itagaki, *Biochim. Biophys. Acta*, 31 (1959) 294.
[10] A. Nason, in W. D. McElroy and B. Glass, *Inorganic Nitrogen Metabolism*, The Johns Hopkins Press, Baltimore, U.S.A., 1956, p. 109.
[11] D. Keilin and E. F. Hartree, *Proc. Roy. Soc. London*, B124 (1938) 397.
[12] M. E. Pullman, A. San Pietro and S. P. Colowick, *J. Biol. Chem.*, 206 (1954) 129
[13] H. J. Evans and A. Nason, *Plant Physiol.*, 28 (1954) 233.
[14] W. J. Lightbown and F. L. Jackson, *Biochem. J.*, 58 (1954) xlix.
[15] C. Iida and K. Yamasaki, *Biochim. Biophys. Acta*, 44 (1960) 352.
[16] C. Iida, *J. Spectroscopical Soc. Japan*, 8 (1960) 141.
[17] T. Torii, *Bull. Chem. Soc. Japan*, 76 (1955) 328, 333.
[18] K. Yagi, *Biochemistry of Flavins*, Kyoritsu Co., Ltd., Tokyo, 1957.
[19] O. Warburg and W. Christian, *Biochem. Z.*, 298 (1939) 150.
[20] F. Moss, *Australian J. Exptl. Biol. Med. Sci.*, 32 (1954) 571.
[21] S. Taniguchi and M. Kono, *J. Biochem.*, (1960) in the press.

[22] D. J. D. Nicholas and H. M. Stevens, in W. D. McElroy and B. Glass, *Inorganic Nitrogen Metabolism*, The Johns Hopkins Press, Baltimore, U.S.A., 1956, p. 178.
[23] D. J. D. Nicholas and A. Nason, *Plant Physiol.*, 30 (1955) 135.
[24] W. D. McElroy and B. Glass, *Inorganic Nitrogen Metabolism*, The Johns Hopkins Press, Baltimore, U.S.A., 1956, chapter VI.
[25] W. W. Westerfeld, D. A. Richert and R. J. Bloom, *J. Biol. Chem.*, 234 (1959) 1889.
[26] W. W. Westerfeld, D. A. Richert and E. S. Higgins, *J. Biol. Chem.*, 234 (1959) 1897.
[27] M. G. P. J. Warringa, O. H. Smith, A. Giuditta and T. P. Singer, *J. Biol. Chem.*, 230 (1958) 97.
[28] M. G. P. J. Warringa and A. Giuditta, *J. Biol. Chem.*, 230 (1958) 111.
[29] E. Itagaki and S. Taniguchi, manuscript in preparation.

INHIBITION OF ACETYLCHOLINESTERASE BY CETYLTRIMETHYLAMMONIUM BROMIDE

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SUMMARY

Employing the Warburg manometric method of assay, the effect of cetyltrimethylammonium bromide on acetylcholinesterase activity was studied. Cetyltrimethylammonium bromide was found to possess anticholinesterase properties; kinetic analysis together with the results from dialysis experiments show that the inhibition is mixed, there being both reversible and irreversible aspects of the total inhibition; the extent of the irreversible inhibition increases with increasing time of incubation of the enzyme with cetyltrimethylammonium bromide. It is concluded that it is unlikely that the inhibition of acetylcholinesterase by cetyltrimethylammonium bromide underlies the previously observed *in vivo* blockade of impulse conduction in whole nerve.

INTRODUCTION

It has been demonstrated that nerves which were appropriately treated with CTMB are rendered especially susceptible to reversible conduction block by a variety of externally applied lipid-insoluble quaternary ammonium ions[1]. If the conditioning exposure to CTMB is too long, however, then complete irreversible conduction block of whole nerve occurs due to the CTMB itself; exposure to CTMB for shorter periods or to lower concentrations produces partial block (*i.e.* total block of a portion of the constituent fibers in a nerve trunk) which is also irreversible[2]. Several questions arise: What is the action of CTMB on nerves which renders them so susceptible to reversible block by curare, acetylcholine, prostigmine, etc.? What is the cause of the irreversible conduction block produced by CTMB itself? Are the two phenomena separate and distinct or do they simply represent two aspects of a continuum of action of CTMB?

This paper deals with an examination of the effect of CTMB on acetylcholinesterase in order to assess one possible mechanism of CTMB-induced block of impulse conduction in nerve. It is reasonable to suspect that CTMB might possess anticholinesterase activity, since many quaternary ammonium compounds are inhibitors of the enzyme. Certainly if CTMB is not an anti-cholinesterase, then the mechanism whereby it produces conduction block cannot be due to inhibition of this enzyme.

Abbreviations: CTMB, cetyltrimethylammonium bromide; DFP, diisopropylfluorophosphate.

If, on the other hand, CTMB is an anticholinesterase, the mechanism of block could be the inhibition of the enzyme, if CTMB is an irreversible inhibitor. It is unlikely, however, that the mechanism involves inhibition of the enzyme if CTMB is a reversible inhibitor, for conduction block produced by CTMB is irreversible.

METHODS

Employing the Warburg manometric technique of AMMON[3], the rate of hydrolysis of acetylcholine was determined by measurement of the CO_2 evolved from a bicarbonate buffer at 37°. Readings were taken every 15 min after gassing with 95 % N_2 and 5 % CO_2 and thermal equilibration. The fluid volume of each flask was 3 ml and the final reaction mixtures comprised NaCl (0.13 *M*), $MgCl_2$ (0.04 *M*), $NaHCO_3$ (0.025 *M*), gelatin (0.1 %, w/v), and 1.25 units of a preparation of acetylcholinesterase derived from bovine erythrocytes (Nutritional Biochemicals Corp.). Acetylcholine chloride (Merck) was used as the substrate, and its final flask concentration was varied from $4.59 \cdot 10^{-3}$ to $1.15 \cdot 10^{-3}$ *M*. Final CTMB concentrations were varied from 10^{-4} to $5 \cdot 10^{-5}$ *M*.

RESULTS AND DISCUSSION

Table I shows some of the results in terms of per cent inhibition; similar data on two other anticholinesterases have been included for comparison. It is apparent that CTMB inhibits acetylcholinesterase, though its potency on a molar basis is far less than for such agents as prostigmine, DFP, and a variety of other tertiary and quaternary ammonium and organophosphorus compounds.

TABLE I

INHIBITION OF ACETYLCHOLINESTERASE

Inhibitor	*Concn.* $(M \times 10^{-5})$	*Acetylcholine concn.* $(M \times 10^{-3})$	*Inhibition* (%)
CTMB	5.0	4.59	29
CTMB	6.0	4.59	34
CTMB	6.0	1.84	51
CTMB	9.0	4.59	68
CTMB	9.0	1.84	75
CTMB	10.0	3.06	84
Prostigmine*	0.1	3.30	50
DFP*	0.125	3.30	50

* From AUGUSTINSSON AND NACHMANSOHN[4] employing acetylcholineesterase prepared from the electric organ of *Electrophorus electricus*.

Fig. 1 is an analysis of the kinetic data by the procedure of LINEWEAVER AND BURK[5]. As indicated by the convergent trend of the three curves at the ordinate, the relative degree of inhibition decreases as the ratio of acetylcholine/inhibitor increases. However, the maximum velocity (from the $1/V$ intercept) is less for the CTMB curves than for the uninhibited reaction, and it is least for the higher concentration of CTMB ($9 \cdot 10^{-5}$ *M*). Since the three curves do not give the same maximum velocity (converge to the same $1/V$ intercept) it appears that the inhibition of acetylcholinesterase by CTMB is not a case of simple reversible inhibition. An extrapolation of the uninhibited and inhibited curves to the $1/S$ intercept reveals that they do not converge there either. According to kinetic analysis of enzyme inhibition[6], this latter point (failure of all three curves to converge at $-1/K_m$) means that the inhibition of acetylcholinesterase by CTMB is not a case of simple irreversible inhibition. Thus the kinetic analysis, utilizing the technique of Lineweaver and Burk, indicates that the inhibition studied is neither simply reversible nor simply irreversible.

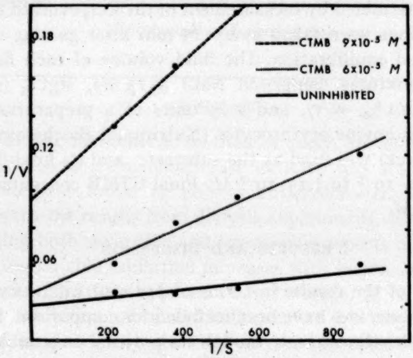

Fig. 1. Inhibition of acetylcholinesterase by CTMB. $1/V$ is the reciprocal of μl of CO_2/15 min (corrected for autolysis). $1/S$ denotes the reciprocal of the molarity of acetylcholine.

Dialysis experiments were conducted in order to amplify upon the apparent simultaneous occurrence of reversible and irreversible inhibition of acetylcholinesterase by CTMB. Four aliquots of equipotent enzyme solution were employed: (a) the uninhibited control, containing no CTMB, (b) the inhibited control, containing 10^{-4} *M* CTMB and not dialyzed, (c) the short-term incubated experimental, containing 10^{-4} *M* CTMB and incubated 10 min at room temperature prior to dialysis (15 h), and (d) the long-term incubated experimental, containing 10^{-4} *M* CTMB and incubated 10 min at room temperature and then 19 h at 10° prior to dialysis (20 h). Data from the manometric analysis of enzyme activity reveal that extensive dialysis restored 78 % of the activity of the enzyme, (c), which had been very briefly exposed to CTMB and 38 % of the activity of aliquot (d) which had been incubated with CTMB for a prolonged period.

The kinetic analysis of the initial experiments together with the results of the dialysis experiments support the conclusion that the inhibition of acetylcholinesterase by CTMB is of the mixed type, there being both reversible and irreversible inhibition occurring simultaneously.

The results, showing a mixed type of inhibition, might have been anticipated from a consideration of the CTMB molecule. The molecule consists of a cationic, quaternarized ammonium "head" and a long-chain, aliphatic cetyl "tail"—found in many surface-active agents possessing cytolytic and germicidal properties[7]. Wilson[8] has demonstrated the reversible, competitive inhibition of acetylcholinesterase by simple alkylated ammonium ions; thus, CTMB might have been expected to exert a degree of reversible inhibition of the enzyme, as our kinetic analysis suggests and the dialysis experiments verify. On the other hand, the presence of the cetyl group might have led us to predict an element of irreversible inhibition by virtue of the known protein denaturing action of many surface-active agents[9].

Regardless of the intimate details of the irreversible* portion of the inhibition of acetylcholinesterase by CTMB, the pertinent fact is that a substantial degree of the total inhibition can be reversed by dilution or dialysis. This fact seems incompatible with the possibility that the complete, irreversible block of conduction in whole nerve by CTMB, *per se*, could be due solely to inhibition of the endogenous axonal acetylcholinesterase; for the nerve block, if not completely, is nearly irreversible**.

Thus the mechanism whereby CTMB blocks impulse conduction must be something more than the mere inhibition of acetylcholinesterase. We are currently examining, electronmicroscopically, the morphological effects of CTMB, *per se*, on nerves and their myelin investments. It has already been speculated that CTMB (and, presumably, other surface-active agents) disrupts cell membranes in a nerve trunk[2]. It has been suggested that Schwann investments are first attacked and then the neuronal membranes themselves. The first sites of attack are associated with the conditioning of nerves to subsequent block by externally applied lipid-insoluble quaternary ammonium ions, and the attack on the neuronal membranes is associated with the eventual conduction block produced by CTMB itself. Presumably the reversible block by acetylcholine, curare, etc., in CTMB-treated nerves is due to a reversible reaction with the membrane receptors.

REFERENCES

[1] R. R. WALSH AND S. E. DEAL, *Am. J. Physiol.*, 197 (1959) 547.
[2] R. R. WALSH AND S. E. DEAL, *Biochim. Biophys. Acta*, 31 (1959) 299.
[3] R. AMMON, *Pflüger's Arch. ges. physiol.*, 233 (1933) 486.
[4] K. AUGUSTINSSON AND D. NACHMANSOHN, *J. Biol. Chem.*, 179 (1949) 543.
[5] H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
[6] M. DIXON AND E. C. WEBB, *Enzymes*, Academic Press, Inc., New York, 1958, p. 62.
[7] C. A. LAWRENCE, *Surface-Active Quaternary Ammonium Germicides*, Academic Press, Inc., New York, 1950, p. 66.
[8] I. B. WILSON, *J. Biol. Chem.*, 197 (1952) 215.
[9] F. W. PUTNAM, *Advances in Protein Chemistry*, Vol. IV, Academic Press, Inc., New York, 1948, p. 80.

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* By irreversible we connote a non-reversibility of the enzyme activity by conventional dilution and dialysis techniques.

** In our experience with nerves (frog sciatic) the block is not reversed after 2 h of washing. However, there is the possibility that there could be a reversal of up to 6% that might go undetected, due to a limitation in our techniques of action potential recording[1,2]. This possible 6% reversal is to be compared with the 35-40% restoration of enzyme activity after dialysis following incubation of the enzyme with CTMB for periods of time far exceeding the times of exposure of whole nerves to CTMB.

STUDIES ON CHOLINE DEHYDROGENASE*

III. ORGANIZATION OF THE RESPIRATORY CHAIN IN CHOLINE OXIDASE

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SUMMARY

1. Titration of rat-liver mitochondria with azide, antimycin A, 2-*n*-heptyl-4-hydroxyquinoline N-oxide, and cyanide showed quantitatively very different effects on succinic and on choline oxidase activities. The former enzyme system was very much more sensitive to each of these inhibitors and reached complete inhibition at relatively low concentrations of these inhibitors; the latter showed a significant activity even at high concentrations of all the inhibitors except cyanide.

2. While choline oxidase was partially resistant to antimycin A and quinoline oxide, the reduction of cytochrome *c* by choline was completely inhibited.

3. Added cytochrome *c* stimulated succinate oxidation several fold without affecting choline oxidase.

4. Anaerobically, no oxidation of choline by fumarate could be demonstrated.

5. In untreated rat-liver mitochondria the succinic and choline oxidase and the corresponding cytochrome *c* reductase activities are additive, not competitive. When the amount of active cytochrome a_3 was limited by titration with azide, the competition of succinic and choline dehydrogenases for the respiratory chain could be demonstrated. Limitation of the rate of the cytochrome *b* to c_1 step by antimycin A or quinoline oxide, however, failed to produce competition between the two oxidases.

6. The differential effects of respiratory chain inhibitors and of added cytochrome *c* were shown to be due to the greater activity of succinic oxidase, as compared with choline oxidase. Following equalization of the activities of the two enzyme systems by titration with malonate these differences disappeared largely, but not entirely.

7. The conditions necessary for the development of the inhibitor-resistant oxidation of choline have been studied. With excess azide present, choline and succinic oxidases compete for the azide-resistant, autooxidizable component. With excess antimycin A or quinoline oxide present, however, succinate oxidation is completely abolished, but significant choline oxidase activity remains.

Abbreviation: DPNH, reduced diphosphopyridine nucleotide.

[*] A part of this work was presented at the 50th annual meeting of the American Society of Biological Chemists, Atlantic City, April 17, 1959. A preliminary note has been published[1].

[**] On leave of absence from the Department of Biochemistry, St. Paul's University, Ikebukuro, Tokyo (Japan).

8. On the basis of these observations, a scheme has been presented for the interrelations of the choline and succinic oxidase chains in liver mitochondria. It is proposed that the two oxidases operate through respiratory chains which are interlinked between cytochrome c_1 and O_2, but not at the oxidation level of cytochrome *b*. It is suggested that the *b* component of the choline chain may become autooxidizable under suitable conditions and thus account for the incomplete inhibition of choline oxidase by respiratory chain inhibitors.

INTRODUCTION

In a recent publication from this laboratory[2], by means of a kinetic approach, the question was explored as to whether a common or separate and independent chains serve succinic and α-glycerophosphoric dehydrogenases in brain mitochondria. It was concluded that a common respiratory chain serves the two dehydrogenases from cytochrome *b* to O_2, or, if the two enzymes are linked to separate cytochrome chains, that a crossover must occur between the chains in the course of normal electron transport. Similar conclusions have been reached independently by WU AND TSOU for the interrelation of succinic and DPNH oxidases in heart muscle preparations[3].

As briefly reported in an abstract[4], when the same kinetic approach was applied to the study of the relations of choline and succinic oxidases in rat-liver mitochondria, the results did not seem to be readily reconcilable with the operation of a common cytochrome chain in the two enzyme systems. Thus, titrations with respiratory chain inhibitors had quantitatively very different effects on succinic and choline oxidase activities; external cytochrome increased the activity of the former several fold without affecting the latter; no competition of the two enzymes for the respiratory chain could be demonstrated; and, anaerobically, no oxidation of choline with fumarate as electron acceptor was observed.

The present paper is a detailed account of this work and of further experiments which lead to an explanation of these observations. It is concluded that functionally interlinked respiratory chains serve choline and succinic dehydrogenases, but the cytochrome *b* moiety serving the two enzymes may be unique to each system.

MATERIALS AND METHODS

The materials and experimental procedures used were as described in previous papers[2,5]. Except as noted, mitochondria were used immediately after preparation.

RESULTS

Titration with respiratory chain inhibitors

The observations which initiated the present investigation are summarized in Figs. 1 to 3 and in Table I. Titration of rat-liver mitochondria with azide or quinoline oxide[*] (Fig. 1) shows a much greater sensitivity of succinic than of choline oxidase toward these inhibitors. At concentrations of these inhibitors which abolish succinate oxidation almost completely, the respiration on choline is little or not at all affected.

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[*] The abbreviation quinoline oxide denotes 2-*n*-heptyl-4-hydroxyquinoline N-oxide.

TABLE I

COMPARISON OF THE EFFECTS OF CYANIDE ON CHOLINE AND SUCCINIC OXIDASES DURING INITIAL REACTION PERIOD

Conditions: Manometric assays during initial 5 min after addition of the substrate. Each vessel contained 16 mg sucrose mitochondria (biuret basis) in 0.01 M KCl–0.015 M phosphate, pH 7.6, 0.5 mg cytochrome *c*, and 0.02 M succinate or 0.017 M choline. Temperature, 38°.

Cyanide concentration (M)	*O_2 uptake*		*Inhibition*	
	Succinate µl	*Choline* µl	*Succinate* %	*Choline* %
None	118.7	48.8		
$3.3 \cdot 10^{-5}$	71.6	44.0	39.7	9.9
$6.7 \cdot 10^{-5}$	66.9	43.3	43.6	11.3
$1.3 \cdot 10^{-4}$	35.7	30.5	70.0	37.5
$3.3 \cdot 10^{-4}$	15.4	9.1	87.0	83.4
$6.7 \cdot 10^{-4}$	6.4	4.3	94.6	91.2

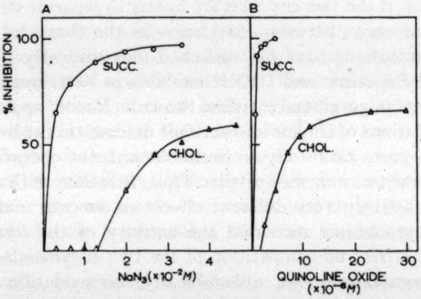


Fig. 1. Titration of succinic and choline oxidase activities of rat-liver mitochondria with azide and quinoline oxide. Manometric determination of O_2 uptake at 30°. Each Warburg vessel contained 150 µmoles phosphate, pH 7.6, sucrose mitochondria in the amounts indicated below, 1.25 mg cytochrome *c*, and inhibitor as indicated in the main compartment, 45 to 50 µmoles choline or 60 µmoles succinate in the side arm, in a total volume of 3 ml. The contents of the side arm were tipped after 7 min temperature equilibration. Activities were calculated from the linear rate obtained between 5 and 20 min after the start of the reaction. In comparing the effects of inhibitors on the two enzymes it was necessary to keep the ratio of inhibitor to mitochondrial protein constant. The much higher activity of succinic than of choline oxidase caused considerable difficulty in meeting this requirement. The problem was circumvented in this and subsequent experiments by using a fixed concentration of mitochondria in all vessels where inhibitor was present, but the activity of the uninhibited succinic oxidase control was calculated from measurements at lower concentrations of mitochondria. Thus, in Fig. 1A all vessels contained 18.4 mg mitochondrial protein (biuret basis) and in Fig. 1B 16.1 mg protein, except the succinic oxidase controls, which contained 5.5 and 3.2 mg protein in experiments A and B, respectively.

Further, while the former enzyme system is completely inhibited at low concentrations of quinoline oxide and over 96 % inhibited by 0.03 to 0.04 M azide, the inhibition of the latter system does not reach completion, and a significant respiration remains which is insensitive to these agents. As shown in Fig. 2, similar results were obtained with antimycin A as inhibitor. In titrations with cyanide, the inhibition of both enzyme systems was virtually complete, but at relatively low concentrations of cyanide the much greater sensitivity of succinic oxidase than of choline oxidase was again apparent.

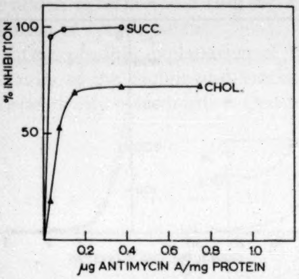


Fig. 2. Titration of succinic and choline oxidase activities with antimycin A. Experimental conditions, as in Fig. 1, except that the temperature was 38° and the amount of mitochondrial protein 10.7 mg in each vessel. The activities were corrected for the slight inhibition produced by the ethanol used as solvent for the antimycin A.

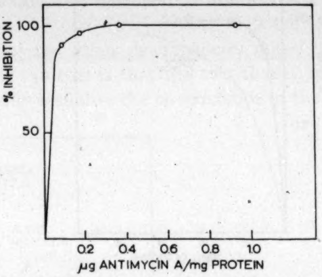
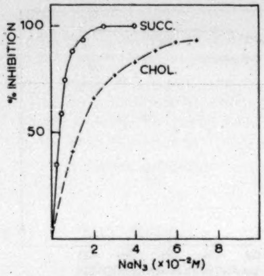


Fig. 3. Titration of choline–cytochrome c reductase activity with antimycin A. The experiment was conducted with a Process and Instruments Company Model RS-3 recording spectrophotometer, thermostated at 30°. Each cuvette contained 150 μmoles phosphate, pH 7.6, 30 μmoles HCN, 100 μmoles choline, 2 mg cytochrome c, 8.6 mg mitochondrial protein, and inhibitor as indicated in a total volume of 3 ml. All components except cytochrome c were preincubated until temperature equilibrium was reached and the reaction was started by the addition of the latter. Activity was calculated from the increase in O.D. at 550 mμ and was corrected for the inhibition caused by the ethanol in which the antimycin was dissolved.

In contrast to the incomplete inhibitions observed with antimycin A and quinoline oxide when O_2 was the electron acceptor (Figs. 1 and 2), when added cytochrome c served as the terminal oxidant the inhibition of choline oxidation was complete, although at low concentrations of these inhibitors the greater sensitivity of succinic oxidase was readily demonstrable. Comparison of Figs. 2 and 3 shows that at a concentration of antimycin A which inhibits the choline-cytochrome c reaction completely (0.4 μg/mg protein), the choline–O_2 reaction is maintained at about 30 % of the uninhibited rate.

In Figs. 1 and 2, inhibition was calculated from the linear part of the reaction rate (5 to 20-min interval after addition of the substrate), since during the initial 5 min at 30° or 38° the rates of both succinate and of choline oxidation increased markedly. The reason for the increase in the succinic oxidase activity with time is probably the previously described activation process[6], while the rate of choline oxidation increases during the initial period because of the limited permeability of mitochondria to choline, as discussed below. If one compares the response of succinic and choline oxidases to azide (Fig. 4), quinoline oxide (Fig. 5), or antimycin during this initial 5-min reaction period, it is found that both enzymes are completely or nearly completely inhibited, but succinic oxidase is still significantly more sensitive to these inhibitors, particularly at low inhibitor concentrations. Thus, the resistance of choline oxidase to respiratory chain inhibitors observed during the linear reaction period develops with time, and its extent depends on the ionic composition and the physiological state of the mitochondria (*cf.* below). In experiments employing devices which measure only the rate during a brief initial period, therefore, some of the differences mentioned would escape notice.



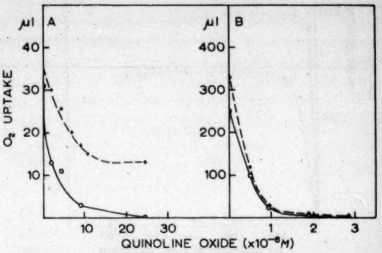


Fig. 4. Extent of inhibition of succinic and choline oxidase activities by azide during the initial phase of oxidation. The experiment utilized a rotating platinum microelectrode connected to a Kin-Tel Model 202B microvoltmeter-amplifier and a Varian Model 11B strip chart recorder. The reaction mixture contained in 2 ml volume 200 µmoles KCl, 30 µmoles phosphate, pH 7.6, 5.1 mg mitochondrial protein, 20 µmoles succinate or 25 µmoles choline, 1.5 µmoles $CaCl_2$, and NaN_3 as indicated, temperature 22°. The reaction was started by the addition of substrate. Rates were calculated from the initial reaction period, which varied from 1 to 5 min depending on the activity of the sample.

Fig. 5. Inhibition of choline oxidase (A) and of succinic oxidase (B) by quinoline oxide during initial 5 min of reaction and during later period. Conditions as in Fig. 1, B. Circles, rate of O_2 uptake during first 5 min after addition of substrate; crosses, rate during 5 to 20 min interval after addition of substrate (linear phase). Rates are expressed as µl O_2 uptake/5 min/ml mitochondrial suspension (= 32.2 mg protein).

Attempts at demonstrating a common respiratory chain

The close interrelations of the succinic and α-glycerophosphoric oxidase chains in brain mitochondria are readily demonstrated by the anaerobic oxidation of α-glycerophosphate by fumarate and by the fact that the oxidations of succinate and α-glycerophosphate are mutually inhibitory whether O_2 or external cytochrome *c* serves as electron acceptor[2]. Attempts to demonstrate a similar relationship between the succinic and choline oxidase chains were not successful. In experiments designed to show the anaerobic oxidation of choline with fumarate as terminal electron acceptor, the formation of the oxidation product, betaine aldehyde, was followed by the 2,4-dinitrophenylhydrazine reaction[7]. Although in control experiments added betaine aldehyde was satisfactorily recovered and a sufficient quantity of mitochondria were employed to detect 0.1 % of the aerobic rate of choline oxidation, no formation of betaine aldehyde was found. Further, in experiments involving the simultaneous oxidation of choline and succinate with either O_2 or cytochrome *c* as electron acceptor, and with a limiting amount of mitochondria present, the rates of oxidation of the two substrates were additive and no evidence for competition was observed.

Other disparities between the choline and succinic oxidase systems were the sensitivity of choline oxidase to amytal[8], and, as discussed in the next section, the stimulation of succinic but not of choline oxidase activity by added cytochrome *c*.

Stimulation by cytochrome c

That rat-liver mitochondria prepared in 0.25 *M* sucrose are partially deficient in cytochrome *c* is evident from the considerable stimulation of succinate oxidation by added cytochrome *c* (Fig. 6). Choline oxidation, however, is unaffected by the addition of cytochrome *c*.

One possible explanation of this and of the other discrepancies noted in the behavior of the choline and succinic oxidase systems is that the two chains operate independently of each other. One could readily visualize the co-existence in the same mitochondrion of a cytochrome chain with a loosely held cytochrome *c* moiety, serving succinic dehydrogenase, and of another chain, serving choline dehydrogenase, in which the cytochrome *c* is more tightly bound. Another explanation of the differential effects of added cytochrome *c* on the two oxidase systems might lie in the much greater activity of succinic dehydrogenase compared with that of choline dehydrogenase and of the consequently greater demand the former enzyme might put on the activity of a common respiratory chain. Thus, the significant concentration of cytochrome *c* retained in liver mitochondria isolated in 0.25 *M* sucrose[8] might suffice to support the full potential rate of choline oxidation, but not of succinate oxidation.

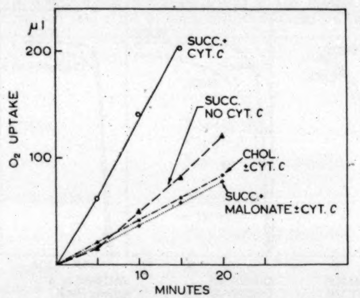


Fig. 6. Effect of added cytochrome *c* on succinic and choline oxidase activities of rat-liver mitochondria. Conditions as in Fig. 1, except as follows: 20 mg mitochondrial protein per vessel; 1.25 mg cytochrome *c* added only where indicated; temperature, 24°. In the lowest curve (+), succinic oxidase activity was depressed with $8.3 \cdot 10^{-4}$ *M* malonate approximately to the level of choline oxidase activity.

In order to decide between these alternatives, succinic oxidase activity was specifically depressed by titration with malonate until it was equal to that of choline oxidase (Fig. 6). Following equalization of the succinic and choline oxidase activities in this manner, the two systems behaved identically, *i.e.*, neither was affected by the addition of cytochrome *c*.

Titration with inhibitors in malonate-treated preparations

Since the experiment just described demonstrated that at least one of the discrepancies in the behavior of the two oxidase systems is due to the relatively higher activity of succinic oxidase, the titrations with respiratory chain inhibitors were repeated with preparations in which the succinic and choline dehydrogenase activities were made approximately equal by means of malonate. As seen in Fig. 7, this treatment brings the titration curves of the two enzyme systems much closer together. The differences in the titration curves of the two oxidases at high inhibitor concentrations, particularly with quinoline oxide, are again related to the inhibitor-resistant oxidation of choline, although this was intentionally minimized in the present experiment by employing a low concentration of phosphate (*cf.* below). Complete coincidence of the titration curves of succinic and choline oxidases with these inhibitors may be obtained, following equalization of the control activities with malonate and with Ca^{++} present to aid penetration of choline, during the initial 5-min interval after addition of the substrate, since the resistance to inhibitors is not manifest during this period.

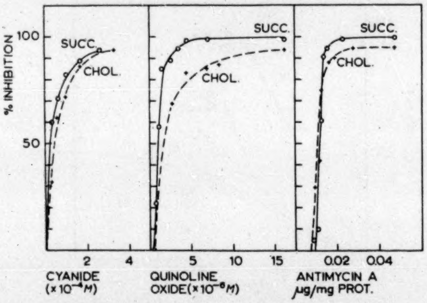


Fig. 7. Comparison of titrations of choline and succinic oxidases with respiratory chain inhibitors following equalization of their activities. Conditions as in Fig. 1 except that the temperature was 28°, and 12.5 µmoles phosphate and 50 µmoles KCl were present per vessel. The succinic oxidase activity was depressed by the addition of malonate to the level of choline oxidase activity. Cyanide experiment, 14.4 mg mitochondrial protein, $1.5 \cdot 10^{-3}$ *M* malonate; quinoline oxide experiment, 10.9 mg protein and $1.5 \cdot 10^{-3}$ *M* malonate; antimycin A experiment, 23.3 mg protein, $1.7 \cdot 10^{-3}$ *M* malonate.

Competition in inhibitor-treated preparations

At this point, it appeared possible that previous failure to demonstrate a competition between the two enzyme systems might have been the consequence of the fact that their combined activities did not exceed the turnover rate of the cytochrome chain in liver mitochondria. If this explanation were correct, and provided that some part of the respiratory chains serving the two dehydrogenases were in common or in intercommunication, the selective suppression of the activities of given members of the cytochrome system should make it possible to demonstrate the competition of the two enzymes. Expt. 1 in Table II shows that when the effective concentration of cytochrome a_3 was reduced by the addition of a low concentration of azide, a competition arose between succinic and choline oxidases, and, as in the competition between succinic and α-glycerophosphoric oxidases[2], the combined rate with both substrates present was less than with the faster one alone. (An explanation of this behavior has been proposed in a previous paper[2].) That the inhibition of succinate oxidation was caused by the operation of choline oxidase and not by choline itself was shown by the fact that the addition of amytal, an inhibitor of choline oxidase, re-established the original rate of succinate oxidation and of fumarate formation. It may be concluded from this experiment that at the oxidation level of cytochrome a_3

TABLE II

COMPETITION OF SUCCINIC AND CHOLINE DEHYDROGENASES FOR THE RESPIRATORY CHAIN

Conditions: Manometric assays at 30°, pH 7.6, in 0.05 M phosphate, with 0.02 M succinate and/or 0.017 M choline present. In Expts. 1, 3, and 4, 0.5 mg cytochrome c was added; and in Expt. 2, succinic dehydrogenase activity was depressed with $5 \cdot 10^{-4}$ M malonate to the level of choline dehydrogenase. The amount of mitochondrial protein (biuret basis) per vessel was 6.3 mg in Expt. 1, 3.8 mg in Expt. 2, and 7.8 mg in Expts. 3 and 4. The results are expressed in each case for a 15-min reaction period and 1 ml mitochondrial suspension (25.5 ± 0.3 mg protein).

Experiment	*Electron acceptor*	*Substrate*	*O_2 uptake* *µatoms*	*Fumarate accumulation* *µmoles*
1	Respiratory chain	Succinate	40.0	36.0
	cytochrome a_3 limiting	Choline	20.0	
	(3.3 mM azide)	Succinate + choline	35.6	28.0
2	Phenazine methosulfate	Succinate	51.2	
		Choline	55.4	
		Succinate + choline	112.0	
3	Respiratory chain	Succinate	22.2	22.8
	cytochrome $b \rightarrow c_1$ limiting	Choline	12.5	
	($9.3 \cdot 10^{-7}$ M quinoline oxide)	Succinate + choline	36.1	24.6
4	Respiratory chain	Succinate	20.2	19.7
	cytochrome $b \rightarrow c_1$ limiting	Choline	11.3	
	(0.11 µg antimycin A)	Succinate + choline	33.4	24.6

the two oxidase chains do not act independently of each other, but are functionally interrelated.

When phenazine methosulfate served as the electron acceptor (Expt. 2, Table II), the rates of oxidation of succinate and choline were additive, as expected from the fact that this dye interacts directly with the two dehydrogenases. The lack of competition was evident whether or not the activities of the two dehydrogenases were equalized with malonate.

Contrary to expectation, however, when the rate of the cytochrome b to c_1 step in the respiratory chain was lowered by treatment with low concentrations of quinoline oxide or antimycin A (Expts. 3 and 4), no competition but, instead, strict additiveness of the two oxidase activities was observed. Fumarate analysis confirmed that the oxidation of choline under these conditions failed to inhibit the oxidation of succinate. It appears from these results that at the cytochrome b to c_1 step in the respiratory chain the flow of electrons from the two dehydrogenases does not follow a common path.

Nature of the inhibitor-resistant respiration

The relevant observations on the nature of the inhibitor-resistant respiration are as follows. At 30 to 38° the full rate of the azide-, antimycin-, and quinoline oxide-resistant oxidation of choline does not develop until about 5 min after the addition of the substrate (Figs. 5 and 8) and at 20 to 25° may take up to 15 min to develop. The inhibitor-resistant oxidation is best observed in the presence of high phosphate concentration and with fresh mitochondria (Table III); in aged or frozen-thawed preparations it is small or insignificant.

While in the presence of an excess of antimycin A or of quinoline oxide an inhibitor-resistant respiration develops, under identical conditions the choline-cytochrome *c* reaction is fully inhibited. In view of this fact and the lack of auto-oxidizability of choline dehydrogenase[5], the resistant respiration appears to be due to the autooxidation of a respiratory chain component acting between the dehydrogenase and cytochrome *c*.

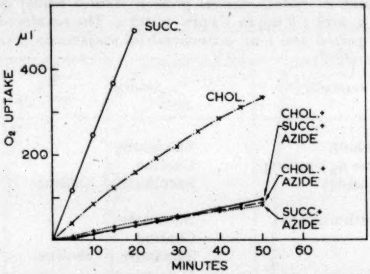


Fig. 8. Competition of succinic and choline dehydrogenases in the azide-resistant respiration of rat-liver mitochondria. Conditions as in Fig. 1, except as follows. Each vessel contained 14 mg mitochondrial protein and 0.5 mg cytochrome *c*, and 150 μmoles azide where indicated. The activities recorded are the actual amounts of O_2 uptake measured.

TABLE III

EFFECT OF EXPERIMENTAL CONDITIONS ON THE AZIDE-RESISTANT RESPIRATION OF LIVER MITOCHONDRIA

Conditions: Manometric determination of O_2 uptake with 0.05 *M* choline as substrate during linear phase of reaction (5 to 20 min after tipping of substrate). Each vessel contained 18.6 mg mitochondria (biuret basis); azide, where present, $3.3 \cdot 10^{-2}$ *M*; pH, 7.6; temperature, 30°.

Mitochondrial preparation	*Rate of respiration* (μl O_2/15 *min*)			
	In 0.05 M phosphate		*In 0.015 M phosphate–0.1 M KCl*	
	No azide	*+ azide*	*No azide*	*+ azide*
Fresh	34·5	14.0	31.0	7·5
After 24 h storage 0°	33.0	6.5	29.0	5·7
After overnight storage in frozen state	31·5	7·5	28.0	4.2

The inhibitor-resistant oxidation of choline has been regularly observed in the presence of excess azide, antimycin A, quinoline oxide, and, to a more limited extent, cyanide. In identical experiments the oxidation of succinate was completely inhibited by antimycin A and quinoline oxide, but not by azide. As illustrated in Fig. 8, the azide-resistant respiration proceeds at the same absolute rate with either succinate or choline or both present as substrate, which would suggest that the same autooxidizable component is limiting the rate in the azide-resistant oxidation of both substrates. Since the activity of succinic oxidase is much greater than that of choline oxidase in the absence of inhibitors, when the same amount of azide-resistant respiration is expressed as a per cent of the activity of the control samples, as in Fig. 1A, the inhibitor-resistant oxidation of succinate is far less significant than that of choline.

The implications of the observations described in this section on the possible identity of the autooxidizable component of the respiratory chain are dealt with in the DISCUSSION.

The effect of Ca^{++}

It was reported by WILLIAMS[9] that in rat-liver mitochondria isolated in 0.25 M sucrose the oxidation of choline is extremely slow or absent, but that following freezing and thawing or the addition of Ca^{++} the apparent permeability barrier to choline is abolished and a rapid oxidation ensues. The observations of WILLIAMS were limited to initial reaction rates since he used a platinum micro-electrode for the measurement of O_2 uptake.

The extremely slow initial rate of choline oxidation in fresh mitochondria and the rapid initiation of O_2 uptake following the addition of $7.5 \cdot 10^{-4}\,M$ Ca^{++} were readily confirmed (Fig. 9). As is apparent from this figure, however, following the initial lag the oxidation of choline begins also in the sample not containing added Ca^{++}, and within 10 min following the addition of choline to fresh mitochondria the rates are equal with and without added Ca^{++}. The rate eventually declines in both samples owing to the accumulation of the inhibitor betaine aldehyde[5].

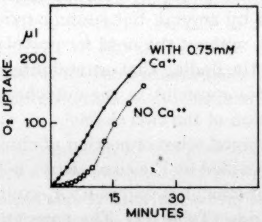


Fig. 9. Rate of appearance of choline oxidase activity in rat liver mitochondria with and without Ca^{++}. Manometric assays at 38°. Each vessel contained 0.8 ml mitochondrial suspension in 0.25 M sucrose (27.7 mg protein) in the side arm, 50 μmoles each of choline, and KCl, and 7.5 μmoles phosphate in the main compartment. Total volume, 3 ml. A 10-min temperature equilibration without shaking preceded the tipping (zero time).

Numerous experiments designed to explore the initial lag in the oxidation of choline in intact mitochondria led to the conclusion that while its underlying cause may be a restricted permeability of the mitochondrial membrane to choline, the phenomenon is a transient one and disappears spontaneously without resorting to conditions which are known to destroy the mitochondrial membrane. Thus, if a relatively concentrated mitochondrial suspension (25 to 30 mg/ml) in 0.25 M sucrose is added to choline-buffer mixture, no O_2 uptake is evident during the first 5 min (Fig. 9), whereas if the mitochondria are preincubated for about 10 min in a more dilute suspension (2.5 to 5 mg/ml) oxidation begins immediately upon the addition of choline. The circumstance that the former technique approximates the conditions apt to be employed in the measurement of respiration with an oxygen electrode, whereas the latter technique parallels the conditions of the manometric assay of choline oxidase employed in this laboratory, may explain why the lag described by WILLIAMS[9] was not encountered in our earlier work[5].

DISCUSSION

The question whether the respiratory chains linking the various dehydrogenases in liver mitochondria to O_2 are the same, completely separate, or separate but interlinked may be at least partially answered on the basis of the experiments presented in this and in the preceding paper[8]. It has been shown that the cytochrome components reduced in the steady state during the oxidation of choline are spectrophotometrically indistinguishable from those operative in the oxidation of succinate and DPNH[8]. The spectrophotometric data presented in the previous paper did not rule out the possibility, however, that the choline, succinic, and DPNH oxidase systems each possess their own cytochrome moieties which are not functionally interlinked. The observations reported in the first part of this paper, in fact, did not appear compatible at first with the operation of a common respiratory chain in choline and succinic oxidases. The quantitative differences noted in the relative response of these two enzyme systems to respiratory chain inhibitors and the qualitative difference in their response to added cytochrome *c* have been satisfactorily explained by the higher activity of succinic oxidase and of the consequently greater demand it puts on the operation of the cytochrome chain. The fact that choline oxidase is completely inhibited by amytal, but succinic oxidase is unaffected by this reagent[8,10], has been explained, without the need for postulating separate respiratory chains for the two enzymes, by the finding that amytal interrupts the flow of electrons from choline dehydrogenase (flavoprotein) to the cytochrome chain, and, therefore, acts prior to the possible junction of the two chains[8].

Direct evidence for a functional interconnection of choline and succinic oxidases at the cytochrome a_3 level is provided by the competitive behavior of the two systems under conditions where the functional cytochrome a_3 content of the mitochondria is limited by titration with azide (Table II). The competition of the two systems for the azide-resistant, autooxidizable component of the respiratory chain (Fig. 8) provides further evidence for their close interrelation.

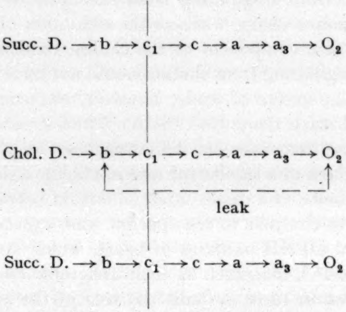
There remain three observations which cannot be readily interpreted if one assumes that all the members of the respiratory chain are in common—or in functional interconnection—in the choline and succinic oxidase systems. The first of these is the failure to demonstrate the thermodynamically feasible anaerobic oxidation of choline by fumarate. Second, when the rate of the cytochrome *b* to c_1 step is lowered with antimycin A or quinoline oxide to a point where the oxidation of succinate is significantly inhibited, the activities of choline and succinic oxidases are still additive, rather than competitive. Third, with a great excess of either of these inhibitors present, the oxidation of succinate is completely abolished, but appreciable choline oxidase activity remains.

A satisfactory explanation of these discrepancies may be offered on the basis of two assumptions: first, that the cytochrome *b* components serving choline and succinic dehydrogenases, respectively, are not the same and are not directly interlinked, and, second, that the component which becomes autooxidizable when the inhibitor-resistant oxidation of choline appears is the cytochrome *b* component of the choline oxidase chain (Scheme 1). In terms of this scheme it may be readily visualized why the two enzyme systems do not compete in the presence of low concentrations of antimycin A or quinoline oxide. These inhibitors, acting between cytochromes *b* and c_1, interrupt the flow of electrons from any dehydrogenase molecule which is attached to one of the respiratory chains which had been inhibited, and there remains a great excess of cytochromes c_1, *c*, *a*, and a_3 to serve those dehydrogenase molecules (choline or succinic), whose cytochrome *b* to c_1 step has not been affected. As to the oxidation of choline but not of succinate in the presence of either of these inhibitors, complete interruption of the *b* to c_1 step would leave no pathway for the electrons originating from succinate to reach the autooxidizable component of the choline chain, whereas the oxidation of choline would still occur to the extent that the *b* component of the choline oxidase chain is autooxidizable, since the electrons originating from choline would not have to pass the *b* to c_1 barrier. In the presence of an excess of azide, however, electrons originating from either dehydrogenase could reach the autooxidizable *b* moiety, those from choline dehydrogenase directly, those from succinic dehydrogenase by the interconnection at the c_1 level. Finally, the lack of a significant rate of choline oxidation by fumarate might be caused by the absence of a direct interconnection between the two chains at the cytochrome *b* level, in contrast to the succinic and α-glycerophosphoric oxidases of brain or succinic and DPNH oxidases of heart, which appear to be interlinked at the cytochrome *b* level[2,3], inasmuch as their anaerobic cross reactions are not antimycin-sensitive[2]. Even in these systems the rate of the anaerobic cross reaction is only 2% of the aerobic activity of the slower of the two interacting oxidases, and the further restriction imposed by the indirect path postulated in Scheme 1 might reduce the rate of interaction of choline and succinic dehydrogenases to an insignificant level.

The reason why choline oxidation in the presence of excess azide, antimycin A, or quinoline oxide is thought to be mediated by a component at the oxidation level of cytochrome *b* is that inhibition by amytal (acting between choline dehydrogenase and cytochrome *b* (see ref. 8)) is complete, whereas a significant level of choline oxidation is observed in the presence of inhibitors which act between cytochromes *b* and c_1. The component which becomes autooxidizable when the inhibitor-resistant respiration develops does not have to be cytochrome *b* itself, of course, but may be an as yet unrecognized electron transport component functioning between the amytal-sensitive site and cytochrome c_1.

Since the early observations of KEILIN AND HARTREE[11], the autooxidizability of cytochromes of the *b* type has been often invoked as an explanation of the inhibitor-resistant respiration of certain tissues, particularly higher plants[12–14]. Not infrequently this explanation has been countered with the "excess oxidase" hypothesis. (For a detailed discussion *cf.* CHANCE AND HACKETT[15].) Applied to choline oxidase, this hypothesis might be paraphrased as follows: since the turnover rate of choline oxidase in the presence of respiratory chain inhibitors is very low compared with the turnover rate of the respiratory chain component being titrated with an inhibitor, if an insufficient excess of inhibitor is used, the small fraction of residual respiratory chain activity might account for the limited rate of choline oxidation. This explanation is clearly not tenable in the present instance, for a variety of reasons, only one of which will be mentioned. Choline oxidase is completely inhibited during the first few minutes following addition of the inhibitor and part of the activity returns gradually, a behavior which strongly suggests the transformation of a respiratory chain component to an autooxidizable form.

Scheme 1 and the discussion presented fail to account for one property of the inhibitor-resistant respiration, namely, that the inhibition of choline oxidase by cyanide is much more complete than by the other inhibitors tested. Although cytochrome *b* is not thought to form a stable complex with cyanide, conceivably in some manner not yet understood cyanide interferes with the transformation of the *b* component of choline oxidase to an autooxidizable form.


Succ. D. → b → c1 → c → a → a3 → O2
Chol. D. → b → c1 → c → a → a3 → O2
leak
Succ. D. → b → c1 → c → a → a3 → O2

Scheme 1. Postulated interrelations of the succinic and choline oxidase chains in rat liver mitochondria. The vertical lines interconnecting the chains are meant to indicate free electron transfer between the chains at one or more points between cytochromes c_1 and a_3.

In the conventional scheme of the mammalian respiratory chain two or more dehydrogenases are pictured as feeding into a single cytochrome chain at some point. In the case of the succinic and choline oxidase chains the bifurcation would occur at the c_1 level. In this type of representation no mechanism is clearly implied for free electron transfer between adjacent chains. Therefore, it would be difficult to see why partial inactivation of the cytochrome a_3 content of a mitochondrion by azide establishes a competition between choline and succinic oxidases. If the sum of the activities of succinic and choline dehydrogenases is less than the turnover rate of a_3 in an intact mitochondrion, as is clearly the case, the addition of azide should merely lower both activities (by eliminating some of the respiratory chains), but the activities of the remaining choline and succinic oxidase chains should remain additive. The postulated scheme, on the other hand, visualizes the co-existence of parallel respiratory chains in liver mitochondria, interlinked, in the case of the choline–succinate pair, at one or more points between cytochrome c_1 and O_2. The competition established between the two enzyme systems by lowering the functional a_3 content of the mitochondria is clearly more compatible with this visualization of the organization in liver mitochondria.

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REFERENCES

[1] T. Kimura and T. P. Singer, *Nature*, 184 (1959) 791.
[2] R. L. Ringler and T. P. Singer, *J. Biol. Chem.*, 234 (1959) 2211.
[3] C. Y. Wu and C. L. Tsou, *Sci. Sinica (Peking)*, 4 (1955) 137.
[4] G. Rendina and T. P. Singer, *Federation Proc.*, 18 (1959) 308.
[5] G. Rendina and T. P. Singer, *J. Biol. Chem.*, 234 (1959) 1605.
[6] E. B. Kearney, *J. Biol. Chem.*, 229 (1957) 363.
[7] D. R. Strength, *Federation Proc.*, 16 (1957) 256.
[8] L. Packer, R. W. Estabrook, T. P. Singer and T. Kimura, *J. Biol. Chem.*, 235 (1960) 535.
[9] G. R. Williams, *Federation Proc.*, 14 (1955) 986.
[10] L. Ernster, O. Jalling, H. Löw and O. Lindberg, *Exptl. Cell Research, Suppl.*, 3 (1955) 124.
[11] D. Keilin and E. F. Hartree, *Proc. Roy. Soc. (London), B*, 127 (1939) 167.
[12] D. S. Bendall and R. Hill, *New Phytologist*, 55 (1956) 206.
[13] H. Lundegardh, *Physiol. Plantarum*, 8 (1955) 95.
[14] D. P. Hackett, *Ann. Rev. Plant Physiol.*, 10 (1959) 113.
[15] B. Chance and D. P. Hackett, *Plant Physiol.*, 34 (1959) 33.

CELL FREE SYNTHESIS OF ORNITHINE TRANSCARBAMYLASE

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SUMMARY

Synthesis of ornithine transcarbamylase activity by four different disrupted preparations of lysozyme protoplasts of *Escherichia coli* W cells was investigated. Whole protoplast contamination was found sufficient to account for all enzyme synthesis observed in water-lysed protoplasts. On the contrary, no contribution of whole protoplasts could be demonstrated for the ATP-dependent ornithine transcarbamylase synthesis observed in preparations disrupted in a Hughes press. Comparison of preparations disrupted by various methods indicated that the most active enzyme synthesis was found in protoplasts disrupted with a liquid-nitrogen-cooled Hughes press.

With such preparations, both ATP and glucose were found to be absolutely essential for the appearance of any new ornithine transcarbamylase, but the high ATP concentration requirement could be replaced by an energy-generating system. Although Mg^{++} was necessary for ornithine transcarbamylase synthesis, no additional stimulation activity was observed with added Mn^{++}. L-amino acid increased ornithine transcarbamylase synthesis in most experiments; however, it was not possible to repress ornithine transcarbamylase formation by arginine in disrupted protoplasts.

Evidence suggestive of an additional requirement of uridine triphosphate, cytidine triphosphate, and guanosine triphosphate for ornithine transcarbamylase formation was obtained with crude disrupted protoplasts. This requirement was augmented in the fractionated ornithine transcarbamylase-forming system.

The observations that polyvinylsulfate, which is a known RNase inhibitor, is a requirement for maximum ornithine transcarbamylase synthesis and added RNase inhibits the same process suggest a role of RNA in ornithine transcarbamylase synthesis.

Preliminary fractionation of the disrupted preparation indicated that the ornithine transcarbamylase-forming system consists of a particulate fraction together with a soluble fraction that can be recovered by acid precipitation.

The significance of these investigations is discussed.

Abbreviations: ATP, GTP, UTP, and CTP, triphosphates of adenosine, guanosine, uridine, and cytidine respectively; ADP, UDP, CDP, and GDP, diphosphates of the same substances; RNase, ribonuclease from bovine pancreas; FDP, fructose 1,6-diphosphate; OTC, ornithine transcarbamylase; Tris, tris(hydroxymethyl)aminomethane; RNA, ribonucleic acid; PEP, phosphoenolpyruvate; and PVS, polyvinylsulfate.

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INTRODUCTION

In a previous report[1] we described the requirements and peculiarities of the synthesis of OTC by whole cells and protoplasts of *Escherichia coli* W. The original observations on the repression of OTC formation by arginine and on the mechanism of enzyme repression in whole cells were made by MAAS AND GORINI[2–5]. The examination of OTC synthesis seemed particularly well suited for the study of *de novo* synthesis of a biologically active protein, since the formation of this enzyme is so easily controlled and its activity is readily measured.

There have been a number of recent observations on the energy-dependent incorporation of [^{14}C]amino acid into protein by various particulate fractions and disrupted preparations of cells and protoplasts of bacteria[6–10]. In addition, GALE AND SPIEGELMAN (see Review by SPIEGELMAN[11]), ROTH AND MAGASANIK[12], BELJANSKI AND OCHOA[13], and NOMURA *et al.*[14] have reported enzyme synthesis or increase in total protein by similar bacterial preparations. Some of these experiments have been difficult to interpret because of the possible contribution of whole cells or protoplasts in the preparations.

We have recently extended our studies on the biosynthesis of OTC to mechanically disrupted protoplasts of *E. coli*. This report describes new requirements for the appearance of OTC activity not found in cells or protoplasts. Evidence is cited against possible contamination by protoplasts as an explanation for the observed levels of synthesis of this enzyme in mechanically disrupted preparations. In addition, preliminary fractionation experiments indicate that both a particulate and a soluble fraction are necessary for OTC synthesis.

EXPERIMENTAL METHODS

Growth of cells and protoplast formation

Escherichia coli, strain W, was grown at 37° to midlog phase in a minimal salts and glucose medium containing arginine to repress OTC formation. Lysozyme and Versene were used to produce protoplasts as described previously[1], except that the medium for protoplast formation contained 0.33 *M* sucrose and 0.03 *M* Tris, pH 8.7. The conversion of cells to protoplasts was never less than 97 % complete as determined by microscopic counts and by viable-cell counts from surface-spread agar plates. Protoplasts were recovered from the lysozyme incubation by centrifugation for 10 min at 6000 × *g* at 15°. Usually about 4 g of protoplasts (wet wt.) were obtained from 8 g of log-phase cells.

Disruption of protoplasts

Water lysates of protoplasts were obtained by complete suspension of protoplast pellets in distilled water at 3°. Usually 40 ml of water was used /g of wet weight of protoplasts. After standing for 20 min at 3° and centrifugation at 6000 × *g* for 10 min to remove residual cells and some of the unlysed protoplasts, the lysed pieces and "ghosts" were recovered by centrifugation at 14,000 × *g* at 0° for 20 min. These water-lysed preparations still contained about 3 % protoplasts as determined microscopically with a Petroff-Hauser bacteria counting chamber.

Mechanically disrupted protoplasts were prepared in three ways.

With French pressure cell[15]: Each gram of protoplasts to be disrupted was suspended in 3.0 ml of 0.33 *M* sucrose containing 0.03 *M* Tris buffer at pH 8.5, at 3°. The suspension was then passed twice through the French pressure cell (American Instrument Co.) at a rate of about 6 ml/min while being maintained under a pressure of 16,000 pounds/in^2 with a Carver hydraulic press. The French cell was precooled at 2° before use. The disrupted preparations contained about 0.1 % of the original protoplasts as determined with the bacteria counting chamber.

With Hughes press at —20°: Protoplasts were suspended as described above. A stainless steel Hughes press[16] (Shandon Scientific Co., Ltd., London, England) was precooled in the deep freeze to — 20°, the protoplast suspension was pipetted into the block, the block was returned to the deep freeze for 20 min, and the protoplasts were then quickly pressed through the block with a Denbeigh No. 4 fly press. The disrupted material, which was collected as an icy slush, was melted before addition to the incubation flasks.

With Hughes press at —197°: Protoplasts were suspended as just described. The Hughes press was precooled in liquid nitrogen until boiling stopped. The suspension was added into the block, the block was again immersed in liquid nitrogen, and the protoplasts were pressed through as soon as possible, as described. The disrupted material was collected as a loose dry powder from the block and was quickly melted at 37° just before addition to incubation flasks. The efficiency of breakage with the Hughes press was greater than that with the French pressure cell. Less than 10^7 protoplasts remained per 10 mg of protein or about 0.01 % of the original protoplasts.

Incubation conditions for OTC synthesis

The standard incubation mixture used for these experiments contained the following in a total volume of 2.0 ml: 660 µmoles of sucrose; 200 µmoles of Tris–HCl buffer, pH 7.6; 5 µmoles of $MgCl_2$; 50 µmoles of KCl; 140 µmoles of glucose; 1.8 mg of polyvinylsulfate; 5 µmoles of ATP; 1 µmole each of UTP, GTP, and CTP; and 100 µg each of L-amino acids (except for Tables IV, VI, and IX where a balanced mixture of amino acids was used). The balanced mixture is representative of the average composition of *E. coli* protein[17] and contained in 2.0 ml the L forms of amino acids as follows: 6 µmoles of alanine, 4.7 µmoles of aspartic acid, 0.8 µmole of cysteine, 5.0 µmoles of glutamic acid, 3.8 µmoles of glycine, 0.48 µmole of histidine, 2.2 µmoles of isoleucine, 3.8 µmoles of leucine, 3.7 µmoles of lysine, 1.6 µmoles of methionine, 1.56 µmoles of phenylalanine, 2.2 µmoles of proline, 2.9 µmoles of serine, 2.2 µmoles of threonine, 0.48 µmole of tryptophan, 1.0 µmole of tyrosine, and 2.7 µmoles of valine.

The incubation mixtures were shaken at 37° in 25-ml Erlenmeyer flasks. An aliquot was then removed from each flask and stored at 1° during the incubation period as separate zero time controls. All flasks were stoppered, incubated for 60 min (or the time indicated), and the reaction was stopped by chilling to 0°.

Assays of OTC

OTC activity was measured as described. The increase in enzyme activity was always calculated as the difference between that observed after the incubation period and the zero time control for each flask. Both whole cells and protoplasts had to be treated with toluene or disrupted by other means to demonstrate full activity. Pretreatment with toluene was not required to measure enzyme activity in disrupted protoplasts or the fractionated preparation. One unit of enzyme activity is defined as 1 μmole of citrulline produced in 1 h under the stated conditions.

Other procedures

Total protein was determined by the method of LOWRY *et al.*[18]. Counts of cells and protoplasts were made with a Petroff-Hauser bacteria counting chamber with a phase-contrast microscope. Cells and protoplasts are easily differentiated by this method as are protoplasts and "ghosts".

MATERIALS

PVS was synthesized according to the method of NOMURA *et al.*[14], using polyvinyl alcohol (98 % hydrolyzed) from Matheson, Coleman, and Bell, Norwood, Ohio. Other materials were obtained from the indicated sources: ATP, GTP, UTP, CTP, ADP, GDP, UDP, and CDP from the Pabst Laboratories, Milwaukee, Wisc. (U.S.A.); the potassium salt of PEP was prepared from the silver barium salt obtained together with pyruvate kinase from C. F. Boehringer and Son, Tutzing, Germany; FDP and lysozyme from Nutritional Biochemicals Corporation, Cleveland, Ohio; L-amino acids from Mann Research Laboratories; 5 × recrystallized salt- and protease-free RNase (from bovine pancreas) from Sigma Chemical Co., St. Louis, Missouri; chloramphenicol from Parke, Davis and Company, Detroit, Michigan.

RESULTS AND DISCUSSION

Apparent OTC synthesis by water lysates of lysozyme protoplasts

Because of the success of other workers in demonstrating enzyme synthesis and amino incorporation into protein by water-lysed preparations of protoplasts, we turned to this type of preparation first in our study. The data of Table I seem to demonstrate cell-free synthesis of OTC in the water lysate. In this experiment $21.3 \cdot 10^8$ whole protoplasts synthesized 76.9 units of OTC or an average of 3.6 units/10^8 protoplasts. Under the same conditions, the water lysate, which contained only $5 \cdot 10^8$ protoplasts and 7 times as much total protein, synthesized 83.8 units of enzyme. The amount of enzyme synthesized was caused, at least in part, by those protoplasts

TABLE I

OTC SYNTHESIS IN WATER LYSATES OF LYSOZYME PROTOPLASTS

Incubations were at 37° for 60 min in a total volume of 2.0 ml.

	Protoplasts	*Lysate*
OTC units/ml	76.9	83.8
Protoplasts × 10^8/ml	21.3	5.0
Calculations		
OTC units/protoplast	3.6	3.6
OTC units synthesized by protoplasts	76.9	18.0
Apparent protoplast-free synthesis of OTC	None	65.8
If synthesis is caused by protoplasts; units/10^8 protoplasts	3.6	16.7

that survived lysis. The contribution these contaminating protoplasts could make was estimated from the amount of OTC synthesized by protoplasts that had not been lysed (Column 1, Table I). Thus it was calculated that $5.0 \cdot 10^8$ protoplasts would have synthesized 18.0 units of OTC. Since 83.8 units actually appeared it would be concluded that cell-free synthesis had produced 65.8 units. But this conslusion rests on the assumption that protoplasts surviving lysis had the same synthetic efficiency as whole protoplasts in the normal reaction mixture. It must be emphasized that protoplasts surviving lysis are in an environment rich in the intracellular components released by the lysed protoplasts and thus may perform more efficiently than protoplasts in a simple defined medium. To test this possibility, $2 \cdot 10^8$ more protoplasts were added to the water lysate, and the synthesis of OTC was measured. This experiment was, in fact, conducted simultaneously with the experiment described in Table I, but for clarity of discussion the data are presented in Table II. We added $2 \cdot 10^8$ fresh protoplasts to the water lysate of Table I that contained $5 \cdot 10^8$ protoplasts. Assuming the synthetic efficiency of whole protoplasts given in Table I, that is 3.6 units/10^8 protoplasts, we would have expected an increase in OTC synthesized by the water lysate from 83.8 to 91.0 if the added protoplasts were operating at the calculated efficiency. However, the observed increase was much higher; we found 130 units instead of the expected 91. Since the increased synthesis in the water lysate in this case is only attributable to the added protoplasts, the data show that protoplasts metabolizing in the presence of the water lysate of other protoplasts have a synthetic efficiency of 23.4 units/10^8 protoplasts rather than the 3.6 units exhibited by whole protoplasts in the standard incubation medium. This increased synthetic efficiency can more than account for the total enzyme synthesis observed in the water lysate, and, therefore, we must conclude that the observed enzyme synthesis is caused entirely by the contaminating protoplasts. The explanation for the observation that the $2 \cdot 10^8$ protoplasts had a synthetic efficiency of 23 as contrasted to an efficiency of 16.7 by $5 \cdot 10^8$ original contaminating protoplasts lies in the possibility that some of these protoplasts had been damaged during water lysis.

TABLE II

OTC SYNTHETIC EFFICIENCY BY PROTOPLASTS ADDED TO WATER LYSATE

Conditions were same as for Table I, except that $2 \cdot 10^8$ fresh protoplasts were added to the water lysate of Table I. See text for discussion.

	Expected	*Observed*
Protoplasts × 10^8	5 + 2	5 + 2
If synthesis is cell free	(83.8 + 7.2) 91	130
Synthesis by added protoplasts	7.2	46.2
Synthetic efficiency of added protoplasts	3.6	23.4

OTC synthesis by mechanically disrupted protoplasts

To obtain more complete breakage of protoplasts under relatively mild conditions, we turned to mechanical disruption methods. Table III is a comparison of results obtained with three different methods of breaking lysozyme protoplasts. In a number of experiments when we used the French pressure cell, the synthesizing activity of the resulting disrupted material was consistently lower than that of preparations

TABLE III

OTC SYNTHESIS BY MECHANICALLY DISRUPTED PROTOPLASTS

Conditions as in Table I. The units synthesized in 90 min are average values obtained from two or more experiments in each case and normalized to OTC units/10 mg of protein

Material	*Units OTC synthesized in 90 min*	*Protoplasts present per 10 mg protein*
Protoplasts	680	$1 \cdot 10^{11}$
Disrupted by:		
French cell (3°)	3	$3 \cdot 10^{8}$
Hughes press (—20°)	6	
Hughes press (—196°)	16.5	$\sim 10^{7}$

made with the Hughes press at —20°, and the observed activity seemed to be a result of residual protoplasts. By lowering the temperature of the Hughes press block to that of liquid nitrogen, in an attempt to reduce denaturation to a minimum during the breakage process, we found that OTC formation was considerably improved in comparison to the other two methods. The number of protoplasts remaining after this procedure was so small that, even if they synthesized OTC at 10 times the normal rate, OTC would not be detected by our assay method. However, from Table II it is obvious that the amount of enzyme synthesized by disrupted protoplasts is quite small relative to that of the whole protoplasts. None of these mechanically disrupted preparations showed any increase in OTC activity in the absence of ATP, whereas this increase of enzyme in protoplasts was essentially unaffected by ATP. Because of the success with the liquid-nitrogen-cooled Hughes press, this method was used in our further investigation of the conditions and requirements for OTC formation.

Requirements for OTC synthesis

Fig. 1 shows the time course of the synthesis of OTC in disrupted protoplasts prepared in the liquid-nitrogen-cooled Hughes press. A comparison of curves 2, 3,

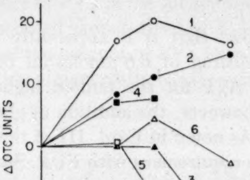

Fig. 1. The time course of OTC formation by protoplasts of *E. coli* W disrupted in a Hughes press. The ordinate is the change in OTC units measured in 2.0 ml of incubation mixture from a zero time activity of 20 units. Incubation conditions: disrupted protoplasts, 12 mg of protein. Curves: ○, complete system; ●, minus UTP, GTP, and CTP; ▲, minus UTP, GTP, CTP, and glucose; ■, nucleoside diphosphates replacing nucleoside triphosphates; □, minus ATP, GTP, CTP, and UTP; and △, complete system plus 100 μg of chloramphenicol.

and 5 indicates that the synthesis of OTC is dependent on the presence of both ATP and glucose. Curve 5 shows the results obtained when the preparation is incubated in the absence of ATP, curve 3 shows the results when the preparation is incubated in the presence of ATP but in the absence of glucose and curve 2 shows the results obtained in the presence of both glucose and ATP. The explanation for the glucose requirement is not evident to us, since, as will be shown later, glucose cannot be replaced by FDP. The glucose effect may be associated in some unknown way with the release of repression. In the absence of either glucose or ATP there was a large destruction of endogenous enzyme evident after 90 min of incubation. For this reason, we did not extend our incubations beyond 90 min in the succeeding experiments. As can be seen in curve 1, the addition of the triphosphates of guanosine, uridine, and cytidine together with ATP and glucose resulted in an increase in OTC synthesis over that with only ATP and glucose (curve 2). This stimulation of synthesis by the three nucleoside triphosphates was not consistently observed with disrupted protoplasts; and the individual contribution of each nucleotide has not been examined. Replacement of the nucleoside triphosphates by the corresponding diphosphates (curve 4) showed a level of OTC formation that is perhaps lower than that obtained with ATP alone. This indicates that the rate of generation of triphosphates by this disrupted preparation in the presence of glucose is insufficient to support maximal enzyme formation. As with cells and protoplasts[1], the appearance of OTC activity in the disrupted protoplasts is similarly inhibited by chloramphenicol, curve 6. This observation suggests that the process of protein synthesis is involved in this enzyme-forming system. None of the additions in the above experiment influenced the measurement of the activity of OTC at the concentrations present in the final reaction mixtures for enzyme assay.

Expt. I of Table IV demonstrates that Mg^{++} is required for OTC synthesis. The requirement for both ATP and glucose is also shown in this experiment. NISMAN[7] found the addition of Mn^{++} was necessary for incorporation of amino acids into protein of lysozyme lysates of *E. coli*. We found, however, that Mn^{++} added with Mg^{++} usually caused a lowering of the amount of OTC formation by disrupted protoplasts (Expt. II, Table IV).

Expt. IV of Table IV shows that it is apparently possible to substitute an energy-generating system consisting of 6.6 μmoles/ml of PEP, 5 μg of pyruvate kinase, and 0.25 μmole/ml of ATP for the tenfold higher concentration of ATP. Even under these conditions, however, the addition of glucose resulted in a marked stimulation of OTC synthesis. As noted in Expt. III of this table, it was not possible to replace the ATP and glucose requirement with FDP. Since a large increase in OTC units was not observed when the generating system was substituted for the high ATP concentration, it may be concluded that this system is not as sensitive to high ATP concentrations as had been found for other bacterial systems[6-8,11]. Fig. 2 shows an experiment in which the effect of ATP concentration on OTC synthesis was examined. This experiment was conducted in the absence of UTP, GTP, and CTP in order to rule out possible transphosphorylations to form ATP. Maximum OTC synthesis apparently takes place at about 5 μmoles/ml of ATP, or about twice the concentration used in the standard incubation mixture in these experiments.

NOMURA *et al.*[14] found that PVS was necessary for maximal appearance of amylase in lysozyme lysates of *Bacillus subtilis*. The action of PVS was attributed to

TABLE IV

REQUIREMENTS FOR OTC FORMATION

The units of enzyme activity shown are the measured increases after 60 min of incubation at 37°. "Complete" incubations contained the standard incubation mixture described in METHODS, except for Exp. I, in which no UTP, CTP, or GTP was added. In Exp. IV, the generating system consists of: 5 μg/ml of pyruvate kinase, 6.6 μmoles/ml of phosphoenolpyruvate, and 0.25 μmole/ml of ATP instead of 2.5 μmoles/ml. 10–12 mg of disrupted protoplast protein were added to each incubation mixture.

Expt.	*Incubation conditions*	*Δ units of enzyme activity/2 ml*
I	Complete	8
	Minus $MgCl_2$	0
	Minus glucose	0
	Minus ATP	0
II	Complete	13
	Minus nucleoside triphosphates	0
	Plus $MnCl_2$, 5 μmoles	7
	Plus $MnCl_2$, 10 μmoles	6
III	Complete	17
	FDP 100 μmoles instead of ATP and glucose	0
	Imidazole buffer, 100 μmoles per ml instead of Tris buffer	11
IV	Complete	17
	Generating system — no glucose	17
	Generating system + glucose	26

its inhibition of the intracellular RNase. As demonstrated by the data of Table V, we also found an enhancement of the appearance of OTC synthesis in the presence of PVS. Therefore this compound was added routinely in all experiments with disrupted protoplasts. In contrast, synthesis of OTC by whole cells or protoplasts never showed any response to the presence of PVS in the incubation medium. As shown in Table V, the RNase activity of disrupted preparations of *E. coli* was inhibited to 50% of the control level by addition of the amount of PVS found to stimulate OTC synthesis. In the absence of PVS, addition of pancreatic RNase completely blocked OTC formation,

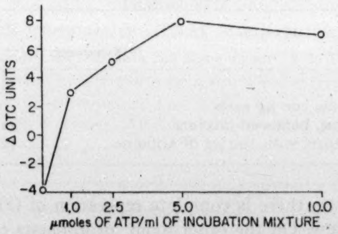

Fig. 2. ATP requirement for OTC synthesis by protoplasts disrupted in a Hughes press. The ordinate is the measured increment of enzyme units formed per 2.0 ml after 60 min of incubation at 37°. Incubation mixtures did not contain UTP, CTP, or GTP. 10 mg of disrupted protoplast protein were added to each incubation mixture.

TABLE V

EFFECT OF POLYVINYLSULFATE ON OTC SYNTHESIS AND RNASE ACTIVITY

OTC formation for whole cells and protoplasts is shown as units synthesized per 1 mg of protein. OTC formation for disrupted protoplasts is shown as units synthesized per 10 mg of protein. RNase activity was measured as release of cold perchloric acid-soluble material absorbing at 260 mμ in the Beckman DU spectrophotometer after 60-min incubation at 37° of 10 mg of disrupted protoplasts. The standard incubation medium was used, and all incubations were at 37° for 60 min in the above experiments, except that triphosphate and amino acids were omitted from the RNase-activity determinations.

Materials	*Control*	*PVS 0.9 mg/ml*	*PVS 1.8 mg/ml*
		OTC activity	
Whole cells	363	313	—
Protoplasts	68	65	—
Disrupted protoplasts	3	13	—
Disrupted protoplasts + added RNase 0.5 mg	0	7	—
		E. coli RNase activity	
Disrupted protoplasts	22.2	11.8	6.2

whereas in the presence of PVS this inhibition was only 50%. These experiments suggest that OTC synthesis is dependent on the presence of intact RNA, as was assumed from other experiments with intact protoplasts[1].

In some experiments, disrupted protoplasts were stimulated by a complete mixture of amino acids minus arginine. The experiments shown in Table VI indicate a considerable variability of this effect, but in most cases demonstrate the superiority of balanced mixture of amino acids over that of a mixture containing equal gram quantities of amino acids. This peculiarity of the amino acid requirement was also evident in the amino acid-incorporation studies with lysed lysozyme protoplasts carried out by Nisman[7]. Connell *et al.*[6] also reported variable results with respect to an amino acid requirement for incorporation of radioactive amino acids into *Azotobacter* particles.

TABLE VI

AMINO ACID STIMULATION OF OTC FORMATION IN DISRUPTED PROTOPLASTS

The units of enzyme activity shown are the increases after incubation at 37° for 60 min. See METHODS for content of the balanced mixture of L-amino acids and the incubation mixture. Disrupted protoplasts: 10 mg of protein.

Conditions		*Units of enzyme activity/2 ml*		
	Experiment:	*(A)*	*(B)*	*(C)*
No amino acids		3	14	3
L-amino acids without arginine, 200 μg each		6	18	4
L-amino acids without arginine, balanced mixture		12	—	7
L-amino acids, balanced mixture, with 200 μg of arginine		12	16	—

As reported previously, there is complete repression of OTC in whole cells with as little as 20 μg of arginine. On the other hand, protoplasts could not be repressed with as much as 200 μg of arginine. This was interpreted as indicating a loss of the ability to concentrate arginine by protoplasts. This interpretation has now been confirmed by Maas[19]. Although about a 30% repression of OTC formation in whole

TABLE VII

EFFECT OF NUCLEOTIDE TRIPHOSPHATES ON OTC SYNTHESIS BY PROTOPLASTS AND DISRUPTED PROTOPLASTS

Units of enzyme are the increases observed after incubation at 37°. $4 \cdot 10^8$ protoplasts were added in (1) and (3). Disrupted protoplasts 14 mg protein.

	Δ units of enzyme activity	
	Minus nucleotides	*Plus nucleotides*
1. Protoplasts	17	13
2. Disrupted protoplasts	0	13
3. Mixture 1 + 2	22	32

protoplasts was observed, from the data in Table VI it is evident that the addition of 200 μg of arginine had no affect on the increase in OTC in mechanically disrupted protoplasts. This suggests either that arginine itself is not the repressor molecule or that even higher concentrations of arginine are necessary to effect its binding to the OTC-forming site.

Contribution of whole protoplasts to synthesis of OTC by the disrupted system

Although the small number of protoplasts remaining in the disrupted preparations seemed to preclude the probability that the increases in OTC observed were attributable to whole protoplasts, an experiment was designed to test this contingency. From the data of Tables I and II it was conceivable that a few whole protoplasts in the presence of disrupted cellular material together with glucose, ATP, UTP, CTP, and GTP could be stimulated to an extraordinary rate of enzyme synthesis. In the experiments shown in Tables VII and VIII, protoplasts, disrupted protoplasts, and a mixture of both were incubated in flasks with and without the triphosphates. $4 \cdot 10^8$ protoplasts, when incubated alone, were slightly inhibited by addition of triphosphates in this experiment (Table VII), whereas the mechanically disrupted protoplasts required the presence of these compounds for net appearance of OTC activity. Calculation from the data obtained by mixing the same quantity of protoplasts with the disrupted preparation (Table VIII) reveals that there was a definite small stimulation of OTC synthesis caused by the added protoplasts. Since the extent of the

TABLE VIII

CELL-FREE SYNTHESIS OF OTC BY DISRUPTED PROTOPLASTS

Conditions and data the same as for Table VII.

	A	*B*	*C*
	Protoplasts	*Disrupted protoplasts*	*A + B*
OTC-synthesized units	13	13	32
Protoplasts × 10^8	4.0	<0.1	4.0
Calculations			
OTC synthesis by added protoplasts	13	—	19
Units OTC/protoplasts × 10^8	3.3	—	4.8
Cell-free synthesis (corrected for protoplast contribution)	—	13	13

stimulation observed was independent of the presence of ATP and other triphosphates, it is concluded that the ATP-dependent OTC formation observed in the disrupted protoplasts cannot be the result of contamination of whole protoplasts in the preparation.

Preliminary fractionation of the OTC-forming system

The data thus far presented suggest that cell-free synthesis of OTC takes place in the disrupted protoplasts system. To reveal further requirements for synthesis, preliminary attempts were made to fractionate the OTC-forming system. Immediately after disruption of the protoplasts in the Hughes press, the crude material was centrifuged twice at 6000 × *g* for 10 min at 0° to remove large pieces and the few remaining whole protoplasts. The resulting supernatant was then centrifuged at 105,000 × *g* for 30 min at 0°. The clear supernatant was removed and the particles were washed in 0.33 *M* sucrose, resedimented at 105,000 × *g*, and suspended in 0.33 *M* sucrose. The 105,000 × *g* supernatant was carefully adjusted to pH 5.1 with cold acetic acid, centrifuged at 6000 × *g* for 10 min at 0°, and the precipitate was resuspended in 0.33 *M* sucrose. The data in Table IX show the effect of this fractionation procedure on OTC formation by this system. Incubation of the washed particles alone resulted in only a small increment of OTC that was apparently independent of added ATP. The pH 5 fraction, when incubated with or without added triphosphates, showed no OTC formation. However, when particles and pH 5 fraction were incubated together, a definite ATP-dependent formation of OTC activity was observed. Further stimulation of the formation of this activity occurred with the addition of UTP, CTP, and GTP. As shown in Table IX, after these particles had been aged for 72 h at 1°, this enzyme-forming ability was completely lost. It seems evident from this fractionation experiment that both a particulate and a soluble component are required to carry out OTC formation. This result corresponds to the experience of many investigators studying protein synthesis in a number of different cellular systems[10, 13, 20, 24]. Further experimental work will be necessary to determine if the sequence of steps, from amino acid activation through transfer RNA to final incorporation into a protein molecule by "ribosomes", studied extensively with the aid of ^{14}C amino acids, takes part in the formation of OTC observed in these experiments.

TABLE IX

FRACTIONATION OF THE OTC-FORMING SYSTEM FROM DISRUPTED PROTOPLASTS

The units of enzyme activity shown are the increases after incubation at 37° for 60 min. Disrupted protoplasts, 10 mg of protein; washed particles alone, 3.5 mg of protein; pH 5 fraction, 7.5 mg of protein. See METHODS for incubation mixture and the text for fractionation procedure. Aged particles were stored for 72 h at 1°.

Material	*Δ Units of enzyme activity per 2.0 ml*		
	Control	*ATP*	*ATP, UTP, CTP, GTP*
Disrupted protoplasts	3.2	15.0	16.0
Washed particles, 105,000 × *g*	2.6	1.2	2.6
pH 5 Fraction	0	0.5	0
Particles + pH 5 fraction	—1.8	5.0	9.2
Aged particles + pH 5 fraction	—1.8	—0.6	0.4

Considering all of the data reported in the foregoing experiments, a number of points should be stressed. First, although the observed increases of OTC activity in all of the experiments with disrupted protoplasts was low, it represents between 1–2 % of the synthetic activity of the protoplast. Since increase in total protein was not consistently observed in these experiments, it is possible that the activity increases noted were attributable to a release of template-bound OTC, perhaps triggered by ATP, rather than the *de novo* synthesis of new OTC molecules during the incubation periods. Such a metabolic release of serum albumin has been observed by Peters[25]. Second, although only the synthesis of OTC has been measured in these experiments, this does not exclude the simultaneous participation of some of the components of the incubation mixture in other reactions, such as template repair or fabrication of other materials related to the enzyme-forming machinery. Last, although the experiment demonstrating an inhibition of OTC formation by chloramphenicol suggests net protein synthesis, the measure of an increase in enzyme activity alone is not an unequivocal demonstration of protein synthesis. This observation must be further substantiated with evidence that amino acids are incorporated into the peptide sequences of this specific enzyme. Thus, in conclusion, it can be said that the evidence to date strongly suggests that a cell-free and protoplast-free system from bacteria, which can be fractionated into two parts, supports formation of OTC that is dependent on both amino acid and energy.

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REFERENCES

[1] P. Rogers and G. D. Novelli, *Biochim. Biophys. Acta,* 33 (1959) 423.
[2] L. Gorini and W. K. Maas, *Biochim. Biophys. Acta,* 25 (1957) 208.
[3] L. Gorini and W. K. Maas, in W. D. McElroy and B. Glass, *The Chemical Basis of Development,* Johns Hopkins Press, Inc., Baltimore, 1958, p. 469.
[4] L. Gorini, *Bull. soc. chim. biol.,* 40 (1958) 1939.
[5] J. H. Schwartz, W. F. Maas and E. J. Simon, *Biochim. Biophys. Acta,* 32 (1959) 582.
[6] G. E. Connell, P. Leugyel and R. C. Warner, *Biochim. Biophys. Acta,* 31 (1959) 391.
[7] B. Nisman, *Biochim. Biophys. Acta,* 32 (1959) 18.
[8] E. F. Gale and J. P. Folkes, *Biochem. J.,* 59 (1955) 661.
[9] E. F. Gale, in G. Tunerall, *Recent Progress in Microbiology,* Almquist and Wiksel, Stockholm, 1959, p. 104.
[10] M. Lamborg, unpublished data.
[11] S. Spiegelman, in W. D. McElroy and B. Glass, *The Chemical Basis of Heredity,* Johns Hopkins Press, Inc., Baltimore, 1957, p. 232.
[12] E. M. Roth and B. Magasanik, *American Chemical Society Abstracts, Division of Biological Chemistry, 1959,* Abstract No. 22.
[13] M. Beljanski and S. Ochoa, *Proc. Natl. Acad. Sci. U. S.,* 44 (1958) 494.
[14] M. Nomura, J. Hosoda and S. Nishimura, *Biochim. Biophys. Acta,* 29 (1958) 161.
[15] C. S. French and H. W. Milner in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology,* Vol. I, Academic Press Inc., New York, 1955, p. 64.
[16] D. E. Hughes, *Brit. J. Exptl. Pathol.,* 32 (1951) 97.
[17] R. B. Roberts, P. H. Abelson, D. B. Cowie, E. T. Bolton and R. J. Britton, *Carnegie Inst. Wash. Publ.,* No. 607 (1955).
[18] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.,* 193 (1951) 265.
[19] W. K. Maas, *Biochem. Biophys. Research Communications,* 1 (1959) 13.
[20] P. C. Zamecnik and E. B. Keller, *J. Biol. Chem.,* 209 (1954) 337.
[21] J. L. Simkin and T. S. Work, *Biochem. J.,* 67 (1957) 617.
[22] G. C. Webster, *Arch. Biochem. Biophys.,* 70 (1957) 622.
[23] S. B. Weiss, G. Acs and F. Lipmann, *Proc. Natl. Acad. Sci. U. S.,* 44 (1958) 189.
[24] J. Mager and F. Lipmann, *Proc. Natl. Acad. Sci. U. S.,* 44 (1958) 305.
[25] T. Peters, Jr., *J. Histochem. Cytochem.,* 7 (1959) 224.

LIVER ALDEHYDE OXIDASE: A FORM OF XANTHINE OXIDASE

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SUMMARY

Pig liver aldehyde oxidase is not specific for aldehydes, but in addition catalyzes the oxidation at reduced rates of hypoxanthine, xanthine and DPNH. The sites of binding of acetaldehyde and hypoxanthine by the enzyme appear to be different. Flavin is a component common to the pathways of oxidation of acetaldehyde and hypoxanthine. Quinacrine inhibits the enzymic catalysis by preventing the reduction of the flavin. The heme component present in preparations of the enzyme does not appear to be involved in the enzymic catalysis.

INTRODUCTION

Previously GORDON *et al.*[1] described the isolation from pig liver of a purified enzyme preparation which catalyzed the oxidation of aldehydes, but was inactive with hypoxanthine or DPNH with methylene blue as electron acceptor. More recently MAHLER *et al.*[2] isolated the enzyme in highly purified form and showed it to be a molybdoflavoprotein. They confirmed the finding that the preparation did not catalyze the oxidation of hypoxanthine or DPNH by methylene blue under the conditions of assay used by GORDON *et al.*[1], but other assay conditions and electron acceptors were not tried by the authors.

In the present report data will be presented to show that pig liver aldehyde oxidase catalyzes the oxidation of hypoxanthine, xanthine and DPNH as well as aldehydes. Evidence for the non-participation of the heme component in the enzymic catalysis will be presented.

METHODS AND MATERIALS

Aldehyde oxidase was prepared from pig liver in highly purified form by modifications of the method of MAHLER *et al.*[2]. Several preparations were subjected to electrophoresis under the conditions described by MAHLER *et al.*[2], and the results were in agreement with those previously published[2] showing only a single maximum. The

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Abbreviations: DPNH, reduced diphosphopyridine nucleotide; FAD, flavin adenine dinucleotide.

preparations were estimated to be over 90 % homogeneous from the symmetry of the peak and calculation of the apparent heterogeneity[3]. The electrophoretic measurements were performed by Mr. R. WADE of the Department of Biochemistry, University of Washington.

The assay systems which were used to determine enzymic activity were based on (a) the reduction of 2,6-dichloroindophenol and ferricytochrome *c* measured spectrophotometrically at 38° as described by MAHLER *et al.*[2], (the assays contained catalase but no alcohol), (b) the reduction of methylene blue measured colorimetrically in Thunberg tubes at 38° according to CORRAN *et al.*[4] and (c) the reaction with molecular oxygen determined by means of an oxygen polarograph as described by CHANCE AND WILLIAMS[5]. In the assay with oxygen the complete system contained 0.4 ml of 0.2 M phosphate buffer of pH 7.5, 2.0 μmoles of substrate, and 0.02 ml of 0.1 % catalase solution in a final volume of 1.0 ml. The reactions were started by addition of an appropriate amount of enzyme (0.2 to 0.4 mg protein) and were carried out at a temperature of 23°. Protein was determined by the biuret reaction[6]. FAD was estimated spectrophotometrically from the ΔE_{450} after reduction of the enzyme preparation with substrate or hydrosulfite using the molecular extinction coefficient of $11.3 \cdot 10^6$ cm²/mole[7].

Silicomolybdate solutions were prepared as described by GLENN AND CRANE[8]. Cytochrome *c*, DPNH and catalase were obtained from the Sigma Chemical Company.

RESULTS

Table I contains a summary of the activities of preparations of aldehyde oxidase with different acceptors and substrates. It is apparent on inspection of the table that the enzyme catalyzed the oxidation of hypoxanthine, xanthine and DPNH as well as acetaldehyde with a variety of electron acceptors. The rate of reaction with

TABLE I

ACTIVITY OF ALDEHYDE OXIDASE WITH DIFFERENT ACCEPTORS AND SUBSTRATES

The rates in parentheses were determined with silicomolybdate solution added to the assay system.

Acceptor	*Substrate*	*Activity (μmoles substrate/mg protein/min) of preparations of aldehyde oxidase*						
		1	*2*	*3*	*4*	*5*	*6*	*7*
Methylene blue 38°	Acetaldehyde	0.52	0.33	0.27	0.44			0.27
	Hypoxanthine	0.03	0.16	0.03	0.09			0.11
Indophenol 38°	Acetaldehyde	0.09	0.17	0.15	0.12	0.11	0.10	0.10
	Hypoxanthine	0.04	0.14	0.06	0.08	0.06	0.11	0.10
	Xanthine	0.02	0.05	0.02	0.03	0.01	0.04	
	DPNH	0.01	0.01	0.01	0.01	0.01	0.02	0.01
Cytochrome *c* 38°	Acetaldehyde					0.02 (0.05)	0.03 (0.07)	
	Hypoxanthine					0.01 (0.01)	0.01 (0.01)	
Oxygen 23°	Acetaldehyde	0.20	0.18	0.28	0.19	0.18	0.19	0.14
	Hypoxanthine	0.03	0.05	0.03	0.03			
	Xanthine	0.02	0.02					

acetaldehyde was in most instances substantially faster than with other substrates. Oxygen and methylene blue appeared to be the most satisfactory acceptors as reaction rates were consistently higher than rates with the other acceptors. It should be noted that rates with oxygen were determined at 23° whereas the other rates were determined at 38°. DPNH was able to serve as a substrate for the enzyme but at a very slow rate. In accord with previous work[1,2] the preparations usually failed to exhibit activity with hypoxanthine when assayed with methylene blue (1.0 μmole) as described by CORRAN[4]. When the system was changed and less methylene blue (0.4 μmole) was used in the assay, rates of reaction were determined for acetaldehyde and hypoxanthine (as shown in Table I) which were in accord with the reaction rates observed with oxygen as acceptor. As previously described[2,8] silicomolybdate increased the rate of oxidation of acetaldehyde by the enzyme 2 to 3 fold when cytochrome *c* was used as acceptor. Rates of oxygen utilization by the enzyme preparations were unaffected by the presence of silicomolybdate, cytochrome *c* or both indicating that the increased rate of cytochrome *c* reduction in the presence of silicomolybdate was not secondary to a decreased rate of reaction of the enzyme with oxygen, and represented a real increase in the amount of acetaldehyde oxidized by the enzyme. However, the rate of reaction of hypoxanthine with the enzyme and cytochrome *c* was not increased by the addition of silicomolybdate.

Table II shows the rates of reaction of preparations of aldehyde oxidase with combinations of acetaldehyde and hypoxanthine as substrates. Sufficient amounts of acetaldehyde and hypoxanthine were used in the assays to saturate the system and give maximum rates of reaction. As shown in the table, reaction rates were significantly higher in all instances but one when both substrates were present in the assay system. In only one instance was the rate with both substrates equal to the sum of the rates with the individual substrates. Acceptors other than indophenol were not used in these studies since the rates of oxidation for acetaldehyde and hypoxanthine with other acceptors were not close enough in value to give significant results.

The difference spectrum (reduced minus oxidized) of a preparation of aldehyde oxidase reduced with acetaldehyde is shown in Fig. 1. An identical spectrum was obtained after reduction with hypoxanthine. No additional decrease in O.D. at

TABLE II

EFFECTS OF COMBINATIONS OF SUBSTRATES ON THE ACTIVITY OF ALDEHYDE OXIDASE WITH INDOPHENOL AS ACCEPTOR

Each value given is the average of duplicate determinations.

Preparation	*Activity (Δ O.D./mg protein/min)*		
	Acetaldehyde	*Hypoxanthine*	*Acetaldehyde plus hypoxanthine*
1	2.9	1.5	4.0
2	2.0	1.1	2.7
3	2.1	1.1	2.7
4	2.0	1.1	2.1
5	1.8	2.1	4.0
Average	2.2	1.4	3.1

450 mμ was noted after reduction by either substrate when hydrosulfite was added to the cuvette, indicating that the flavin was completely reduced by acetaldehyde or hypoxanthine. No flavin reduction was noted after addition of substrate until very low concentrations of oxygen were reached (followed by means of the oxygen polarograph) indicating that during the steady state phase of enzymic catalysis the flavin remained almost completely oxidized.

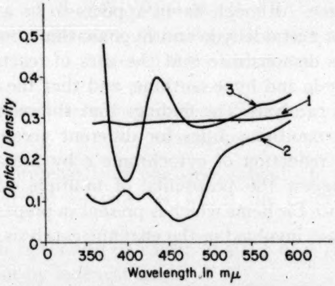

Fig. 1. Difference spectra of aldehyde oxidase: (1) Baseline, (2) enzyme reduced with excess acetaldehyde minus oxidized enzyme, (3) enzyme reduced with acetaldehyde and hydrosulfite minus enzyme reduced with acetaldehyde only. All cuvettes contained 10 mg of enzyme protein, 0.4 ml of 0.2 M phosphate buffer of pH 7.5 and 0.02 ml of 0.1 % catalase solution in final volume of 1.1 ml. To reduce the enzyme 0.02 ml of 1 M acetaldehyde or a small amount (less than 1 mg) of solid hydrosulfite was added to the cuvette.

Quinacrine has previously been shown to be an inhibitor for flavin nucleotide containing enzymes[9] and for aldehyde oxidase[2]. When added to the assay system in concentration 0.001 M, quinacrine inhibited the enzyme catalyzed oxidation of acetaldehyde and hypoxanthine by oxygen to the same degree (approx. 80 %) indicating again that the flavin is a component common to the pathways of oxidation of both hypoxanthine and acetaldehyde. Difference spectra (reduced minus oxidized) of preparations of enzyme reduced by either acetaldehyde or hydrosulfite in the presence of 0.001 M quinacrine showed that only 14 % of the flavin present could be reduced. As stated earlier, 100 % of the flavin was reduced by substrate or hydrosulfite in the absence of quinacrine. It would appear that quinacrine interferes with enzymic catalysis by blocking the reduction of the flavin and thus interfering with the chain of electron transport.

The difference spectrum of aldehyde oxidase reduced by hydrosulfite minus reduced by acetaldehyde is shown in Fig. 1. The spectrum has maxima at 435 and 560 mμ indicating that most of the heme which is present in preparations of aldehyde oxidase[2] is reduced by hydrosulfite but not by acetaldehyde. The finding that the heme is not reduced by substrate suggests strongly that the heme does not participate directly in the enzymic catalysis.

DISCUSSION

Results of the experiments described above suggest that pig liver aldehyde oxidase is a form of xanthine oxidase. The enzyme is not specific for aldehydes, reacting with hypoxanthine and xanthine although at rates appreciably slower than with acetaldehyde. DPNH can also serve as substrate for the enzyme, but at a very slow rate. The finding that hypoxanthine as well as acetaldehyde can completely reduce the enzyme bound flavin indicates that the same enzyme catalyses the oxidation of both substrates and that the oxidation of hypoxanthine is not due to the presence in the preparation of a small amount of a second contaminating enzyme. This is confirmed by the finding that quinacrine inhibits the oxidation of acetaldehyde and hypoxanthine to the same degree. Although flavin appears to be a component common to the paths of reaction for acetaldehyde and hypoxanthine, results of studies using combinations of substrates demonstrate that the sites of reaction with the enzyme are different for acetaldehyde and hypoxanthine, and that the flavin is not the rate limiting component in the catalysis. The findings that the relative rates of reaction for acetaldehyde and hypoxanthine differ for different acceptors and that silicomolybdate stimulates the reduction of cytochrome *c* by acetaldehyde but not by hypoxanthine strongly suggest the possibility of multiple pathways of reaction. Failure of substrate to reduce the heme which is present in preparations of the enzyme indicate that the heme is not involved in the enzymic catalysis.

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REFERENCES

[1] A. H. GORDON, D. E. GREEN AND V. SUBRAHMUNYAN, *Biochem. J.*, 34 (1940) 764.
[2] H. R. MAHLER, B. MACKLER, D. E. GREEN AND R. M. BOCK, *J. Biol. Chem.*, 210 (1954) 465.
[3] E. A. ANDERSON AND R. A. ALBERTY, *J. Phys. and Colloid Chem.*, 52 (1948) 1345.
[4] H. S. CORRAN, J. G. DEWAN, A. H. GORDON AND D. E. GREEN, *Biochem. J.*, 33 (1939) 1694.
[5] B. CHANCE AND G. R. WILLIAMS, *J. Biol. Chem.*, 217 (1955) 383.
[6] A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
[7] E. DIMONT, D. R. SANADI AND F. M. HUENNEKENS, *J. Am. Chem. Soc.*, 74 (1952) 5440.
[8] J. L. GLENN AND F. L. CRANE, *Biochim. Biophys. Acta*, 22 (1956) 111.
[9] E. HAAS, *J. Biol. Chem.*, 155 (1944) 321.

PURIFICATION AND SOME PROPERTIES OF A PROTEASE FROM PEA SEEDS, *PISUM SATIVUM* L, S.SP. *ARVENSE* A. AND G.

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SUMMARY

The purification of a protease from pea seeds (about 350-fold) is described and some properties of the purified enzyme for which the name arvensin is proposed are discussed.

The enzyme acts optimally on casein at pH 8.0; it is inhibited by some salts of heavy metals, but not by iodoacetamide.

It somewhat resembles trypsin. The specific activity of the most pure preparation is about a quarter of that of crystalline trypsin.

INTRODUCTION

During germination seed proteins are broken down into smaller compounds (amino acids and/or peptides) which are then transferred to the growth zones. Proteolytic enzymes from seeds have, however, to our knowledge, not been extensively studied. The degree of purification achieved was low and consequently the resulting preparations were of low activity per unit of protein. The present paper describes the isolation and extensive purification of a protease from pea seeds and some of its properties.

MATERIALS

In all experiments the same batch of non-germinated pea seeds (*Pisum sativum*, s.sp. *arvense* A. and G., race "*aureool*") was used. The seeds were stored in a cool place in the laboratory for more than two years. While the shape, colour and texture showed some changes, the protease content was not appreciably diminished during this period.

The casein (Hammarsten) used was a commercial preparation (Merck; 12.93 % *N* by Kjeldahl method). For use as the substrate, it was always suspended in slightly alkaline water or in buffer solutions, heated at 100° for 30 min with occasional stirring, and then cooled. It was dissolved completely by this treatment. If necessary the solution obtained was adjusted to the appropriate pH. The casein solution could be stored in a refrigerator at 1–4° for at least one week.

Bovine serum albumin was obtained from Poviet Producten N.V., Amsterdam,

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The Netherlands. Its protein content was determined by Kjeldahl analysis, assuming a nitrogen content of 16.0%.

Hemoglobin was prepared in our laboratory from fresh beef blood by the method of M. L. ANSON[1].

The Soy bean trypsin inhibitor (5 × crystall.; Nutritional Biochemicals Corp.) used was found to be highly active against trypsin[2].

Crystalline trypsin was obtained from the Armour Laboratories (Lot No. S92009, 2910 units/mg).

All reagents used were of analytical grade.

Acetone was purified by treatment with $KMnO_4$ and distillation.

METHODS

Determination of activity

Unbuffered 1% casein solutions were always used, except where mentioned otherwise.

The proteolytic activity was measured by the spectrophotometric determination of the TCA-soluble fraction after enzyme action in the following way. A mixture of 5 ml of 1% casein solution of pH 8.0 and 1 ml of a solution of EDTA (0.002 *M* final concentration) adjusted to pH 8.0 was incubated at 38°. After 10 min 2 ml of the enzyme solution were added. 20 min after the addition of the enzyme, 5 ml of an ice-cold 8% TCA solution were added to the reaction vessel and the mixture well shaken and left at room temperature for 30 min. The precipitated protein was removed by filtration through Whatman No. 3 filter paper and the optical density of the clear filtrate measured in a Zeiss PMQ II spectrophotometer at 276 mμ. Zero time and reaction determinations were performed in duplicate.

The proteolytic activity towards casein was expressed in arbitrary units. One unit was defined as that amount of enzyme, which under the assay conditions used, caused an increase in the optical density of the filtrate of 1.000 at 276 mμ and 1 cm light path.

The specific activity was expressed as units/mg of protein as determined by the ROBINSON–HOGDEN modification of the biuret method[3] or by LOWRY's method[4] when the protein concentration of the solution was lower than 0.5 mg per ml. Bovine serum albumin was always used as a standard.

EXPERIMENTAL RESULTS

Purification

The purification procedures developed consisted of the following steps:

Preparation of acetone powder: In order to obtain a stable starting material, free from most of the lipids, pigments and other interfering substances, acetone powder was specially prepared.

The seeds were soaked in excess of deionized water for 24 h at 2°, then homogenized in a Waring blendor in a cold room (2°) with 20 volumes of cold acetone (—20°), followed by immediate filtration by suction. The yellow-brownish powder obtained was freed from adhering acetone in a vacuum desiccator. From 1 kg of seeds about 700 g powder was obtained.

Extraction of the enzyme: 700 g of acetone powder were stirred slowly at 2° for 2 h with 3500 ml of a solution of pH 7.0 containing 0.2 *M* NaCl and 0.001 *M* EDTA. The suspension was filtered through a double layer of cheese cloth and the resulting filtrate was centrifuged at 2° for 50 min at 2000 r.p.m. (radius 25 cm) in an International Centrifuge Model PR-2, head No. 276a. The yellow-brownish supernatant (2400 ml) was decanted (A) and the residue discarded.

Isoelectric precipitation: To solution A, 0.05 *M* HCl (*ca.* 130 ml) was added slowly at 2° with careful stirring to avoid foaming to bring the pH to 6.0. The suspension was centrifuged at 2° and the resulting clear supernatant collected. The precipitate (after redissolving in phosphate buffer of pH 7.0) did not show any proteolytic activity and was discarded (B). The supernatant obtained was adjusted to pH 5.0 by adding again 0.05 *M* HCl (*ca.* 330 ml needed). After centrifuging, the resulting precipitate was redissolved in the minimum volume of phosphate buffer of pH 7.0 at 2° and thus solution C (550 ml) was obtained. The phosphate buffer used contained 0.2 *M* NaCl, 0.03 *M* Na_2HPO_4 and 0.02 *M* NaH_2PO_4[5,6]. On further purification of the supernatant by changing the pH, fractions D and E were obtained which exhibited little and no activity respectively.

First fractionation with acetone: To solution C (20 mg protein/ml) cold acetone (−20°) was added until a final concentration of 15 % (v/v).

TABLE I

PURIFICATION OF PROTEASE

700 g acetone powder were used as starting material. For the determinations and a definition of the activity units, see the "Materials" and "Methods" sections.

Fraction	*Total volume (ml)*	*Protein*		*Activity*		*Specific activity*	*Purification*	*Recovery (%)*
		per ml (mg)	*total (mg)*	*per ml (units)*	*total (units)*			
A (crude extract)	2400	12.2	29280	0.153	367.2	0.013	1	100
B (pH 6 precipitate after redissolving)	490	18.0	8820	0	0	0	0	0
C (pH 5 precipitate after redissolving)	550	19.8	10890	0.580	319.0	0.029	2.2	87
D (pH 4 precipitate after redissolving)	350	4.5	1575	0.063	22.1	0.014	1.1	6
E (pH 4 supernatant)	3650	1.6	5840	0	0	0	0	0
F 0–15 % acetone precipitate (after dialysis and redissolving)	465	22.0	10230	0.075	34.9	0.003	0.2	9
G 15 % acetone supernatant (after dialysis)	745	0.48	357	0.323	240.6	0.673	52	66

During this operation, the temperature of the solution was maintained at or somewhat below —2°. The precipitate after centrifuging at —2° was discarded (F) and the clear supernatant was dialyzed against deionized water at 2° for 30 h, and a clear solution G (745 ml) was obtained.

Lyophilization: As the protein concentration of fraction G appeared to be too low to be suitable for further purification with acetone, the solution was lyophilized. This procedure unfortunately caused some loss of activity, but a stable product was obtained, which could be purified further. The lyophilization product is stable for months when stored in well-closed bottles in a deep-freeze cabinet (—20°). It contained about 15–20% of protein.

Second fractionation with acetone: This was carried out with only part of the powder obtained by freeze-drying. A solution of the lyophilized product was prepared in deionized water with a concentration of 4.5 mg of protein/ml (I). Cold acetone (—20°) was added to give a final concentration of 30% (v/v) in the same manner and the mixture was centrifuged as described before. The inactive precipitate was discarded (II). More cold acetone was added to the supernatant to give a concentration of 50% (v/v). The precipitate obtained after centrifugation showed only slight activity afteı dialyzing (III). Again cold acetone was added to the supernatant to a final concentration of 75% (v/v). After centrifuging the suspension both precipitate and supernatant were dialyzed for 36 h at 2° (fractions IV and V respectively).

TABLE II

FURTHER PURIFICATION OF PROTEASE

As the starting material part of the lyophilized fraction G (see Table I) containing 54 mg protein, was used. The values between brackets in the columns "Purification" and "Recovery" are expressed relative to the crude extract (Fraction A) and thus show total purification and recovery respectively.

Fraction	*Total volume (ml)*	*Protein*		*Activity*		*Specific activity*	*Purification*	*Recovery (%)*
		per ml (mg)	*total (mg)*	*per ml (units)*	*total (units)*			
I redissolved lyophilized fraction G	12	4.5	54.0	2.740	32.9	0.61	1 (47)	100 (60)
II precip. 0–30% acetone, dialyzed;								
supernatant	14	0	0	0	0	0	0	0
precipitate	13	1.7	22.1	0	0	0	0	0
III precip. 30–50% acetone, dialyzed;								
supernatant	9	0.5	4.5	0.045	0.4	0.09	0.1 (5)	1
precipitate	5	1.1	5.5	0.225	1.1	0.21	0.3 (14)	3
IV precip. 50–75% acetone, dialyzed	11	0.35	3.9	1.540	16.9	4.40	7.2 (338)	51 (31)
V 50–75% acetone supernat.; dialyzed	73	traces	traces	0	0	0	0	0

Fraction IV showed the highest activity of all the fractions. The purification was about 7- and 350-fold relative to fraction I and the crude extract A respectively.

The results of the various steps are described in Table I and II.

Properties

Effect of variation in enzyme concentration on protein breakdown: The breakdown of casein by partial purified pea seeds protease (fraction G) of varying concentration is illustrated in Fig. 1. The casein concentration was maintained constant. A linear relationship was obtained. This was not the case, however, when the crude extract A was used, probably owing to the simultaneously occurring splitting of the seed proteins by the enzyme.

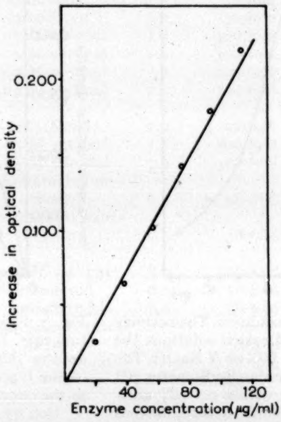

Fig. 1. Effect of enzyme concentration on casein breakdown. Activity determinations were carried out as described in "Methods" section, except without the addition of EDTA. The enzyme concentration was expressed in µg enzyme protein per ml final concentration.

Effect of pH: The pH dependence of the enzymic activity is shown in Fig. 2. The pH optimum appears to be at pH 8.0 and the curve is relatively symmetrical. Measurements below pH 6 were not possible as the enzyme and the substrate precipitated.

Effect of temperature on reaction rate: The rates of protease activity at 20°, 30° and 40° respectively were compared as is shown in Fig. 3.

Substrates: Besides casein, hemoglobin and serum albumin were broken down by this enzyme. In the case of hemoglobin, the enzyme acted upon the native as well as on the heat-denatured form, while with serum albumin only the denatured protein was split to a measurable degree. Another native protein, soy bean trypsin inhibitor, was also attacked by the enzyme (see following section).

In a search for a synthetic substrate, benzoyl-argininamide (0.05 *M*), glycyl-phenylalaninamide (0.05 *M*) and glycyl-glycine (0.05 *M*) were tried, but were not split within 2 h by the most purified enzyme preparation in high concentration (400 µg of enzyme protein/ml of test solution).

Inhibitors and activators: In the following experiments casein solutions were used as the substrate. Salts of heavy metals, notably $CuCl_2$, $HgCl_2$, $FeCl_2$ and $MnCl_2$ inactivated the enzyme, but $FeCl_3$ had no inhibitory effect. The inactivation by $CuCl_2$ could be abolished by addition of EDTA. However, this substance could not restore activity after inactivation by $FeCl_2$ and $HgCl_2$.

Iodoacetamide (IAA) a well-known inhibitor of SH-enzymes, had no significant effect, even when the enzyme was pre-incubated with the inhibitor (3×10^{-2} *M* IAA) at 25° for 30 min before being added to the substrate. On the other hand L-cysteine had a remarkable inhibitory effect, which increased with increasing cysteine concentration.

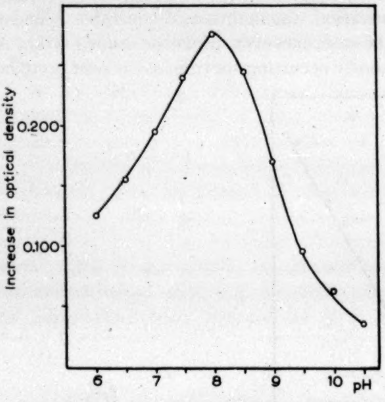

Fig. 2. Effect of pH on casein breakdown. The activity was determined in 1 % unbuffered casein solutions the pH of which was adjusted with *N* HCl or *N* NaOH. The pH was measured by a glass electrode (Radiometer pH meter 24) and remained constant within 0.1 pH units during the enzymic digestion. Blank determinations were carried out at all pH values. A solution of the lyophilized product was used as the enzyme solution (final concentration 63 μg enzyme protein/ml). Activity determinations were carried out without the addition of EDTA.

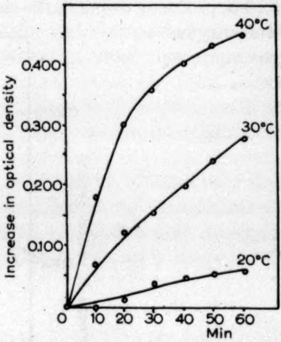

Fig. 3. Effect of temperature on reaction rate. For activity determinations see the "Methods" section. A solution of the lyophilized fraction G was used as the enzyme source (final concentration 63 μg enzyme protein/ml).

As the enzyme showed some resemblance to trypsin, and some beans contain inhibitors of tryptic proteolysis, the effect of crystalline soy bean trypsin inhibitor on the enzyme was investigated. The inhibitor which appeared to be highly active against trypsin, did not only show no inhibition of the protease, but even somewhat activated it when present in 0.01 % final concentration (twice the amount of enzyme protein present). On further examination it was found that soy bean trypsin inhibitor was split by the enzyme to form TCA-soluble, u.v.-absorbing substances more quickly than casein under the experimental conditions used. It should be noted that this inhibitor is quite stable towards denaturation under these circumstances.

The influence of some buffers were also examined when used as solvents for casein. Casein dissolved in phosphate–borate–citrate buffer of pH 8.0[7] was split less than an unbuffered casein solution. The most suitable buffer was a 0.05 *M* veronal–HCl buffer[8]. Casein solutions in this buffer were digested more quickly than the unbuffered

TABLE III

EFFECT OF SOME SUBSTANCES ON THE PROTEASE ACTIVITY TOWARDS CASEIN

For experimental conditions, see "Materials" and "Methods" sections. The phosphate–borate–citrate buffer contained 0.0100 *M* H_3PO_4, 0.0114 *M* H_3BO_3, 0.0067 *M* citric acid, 0.0690 *M* NaOH and 0.0280 *M* HCl.

Added substances and their final concentrations	*Buffer of substrate*	*pH*	*Purity of the enzyme preparation used*	*Enzyme protein concentration per ml of reaction mixture (µg)*	*Relative activity (without addition = 100)*
10^{-4} *M* $CuCl_2$	unbuffered	6.5	58-fold	60	60
$2 \cdot 10^{-4}$ *M* $CuCl_2$	unbuffered	6.5	58-fold	60	32
10^{-3} *M* $CuCl_2$	unbuffered	6.5	58-fold	60	0
10^{-3} *M* $HgCl_2$	unbuffered	7.0	58-fold	60	0
10^{-3} *M* $FeCl_2$	0.05 *M* veronal	7.0	300-fold	70	18
10^{-3} *M* $FeCl_3$	0.05 *M* veronal	7.0	300-fold	50	100
10^{-3} *M* $MnCl_2$	0.05 *M* veronal	7.0	300-fold	75	64
10^{-2} *M* $MnCl_2$	0.05 *M* veronal	7.0	300-fold	75	0
$3 \cdot 10^{-2}$ *M* IAA (pre-incubated)	unbuffered	7.0	58-fold	75	93
10^{-2} *M* L-Cysteine	0.05 *M* veronal	7.0	300-fold	50	80
$2 \cdot 10^{-2}$ *M* L-Cysteine	0.05 *M* veronal	7.0	300-fold	50	10
$4 \cdot 10^{-2}$ *M* L-Cysteine	phosphate–borate–citrate	6.5	58-fold	100	82
$8 \cdot 10^{-2}$ *M* L-Cysteine	phosphate–borate–citrate	6.5	58-fold	100	33
Soy bean trypsin inhibitor (0.01 %)	0.05 *M* veronal	8.0	300-fold	50	170
$2 \cdot 10^{-3}$ *M* EDTA	unbuffered	8.0	58-fold	100	135
$4 \cdot 10^{-3}$ *M* EDTA	unbuffered	8.0	58-fold	100	140
$2 \cdot 10^{-3}$ *M* EDTA	phosphate–borate–citrate	8.0	58-fold	100	100
$2 \cdot 10^{-3}$ *M* EDTA	0.05 *M* veronal	8.0	58-fold	100	100

TABLE IV

INFLUENCE OF BUFFERS ON PROTEASE ACTIVITY TOWARDS CASEIN

For explanation, see Table III.

Buffer used	*pH*	*Purity of the enzyme preparation used*	*Enzyme protein concentration per ml of the reaction mixture (µg)*	*Relative activity (without addition = 100)*
Phosphate–borate–citrate	8.0	58-fold	100	72
0.05 *M* veronal	8.0	58-fold	100	210

substrates. Moreover these solutions appeared to be stable for more than 2 weeks if stored at about 2°.

Tables III and IV show the effects of some inhibitors or activators.

Milk-clotting effect: The lyophilized fraction G (90 µg of enzyme protein/ml test solution) incubated at 38° in the presence of 20 % skimmed milk powder in Tris-buffer (0.05 *M*) of pH 7.5 did not show a noticeable milk-clotting effect within 3 h.

Stability: In general the solution of this enzyme in deionized water at neutral pH could be kept in the frozen state (—20°) for more than two months with only little loss of activity. However, frequent thawing and freezing caused denaturation.

The solution of the enzyme at neutral pH was stable for about one week at 2–4°. It quickly lost activity at pH values below 4 and above 10.

The enzyme was rather sensitive towards heat. Incubation of the purified enzyme solution at 38° for 20 min in the absence of substrate, caused an activity loss of about 60%. Addition of protein substrate stabilized the enzyme towards increase in temperature. This effect will be investigated further.

Storage of the enzyme solution in the presence of toluene caused a considerable loss of activity.

DISCUSSION

The experiments have shown that it is possible to purify a protease from seeds about 350-fold, compared to the crude extract, by the repeated use of isoelectric precipitation and acetone fractionation. As the crude extract contains only a quarter of the total seed protein, the protease described forms about 0.05% of the total protein or less. Its spatial distribution in the seeds has not yet been investigated, but will be considered in further studies.

Some aspects of the purification deserve comment.

It appeared necessary to use acetone powder as the starting material. Acetone apparently removes substances, probably of lipid nature, which interfere with the purification. It also dissolves part of the colouring material which can interfere with spectrophotometric measurements.

The lyophilization step, though time-consuming, was also necessary in order to obtain a stable product.

It is of advantage to carry out the second acetone fractionation in the stepwise manner described. Direct fractionation by adding acetone in one step until 50% (v/v) yielded products with appreciably lower specific activities. This phenomenon is still unexplained; it might be due to protein–protein interaction.

A combination of the procedure described with ammonium sulfate fractionation did not give satisfactory results. Fractionation of the crude extract with ammonium sulfate yielded most of the enzyme activity in the 40% saturation precipitate, but caused heavy losses of activity.

Calcium gel adsorption methods have also proved unsuccessful up-to-now.

Further purification of the enzyme will be attempted by ion-exchange chromatography. We have no unambiguous indications about the state of purity of the preparation obtained. Paper electrophoresis did not appear to be suitable.

For an explanation of the curious effects of some metals further experiments will be necessary. We therefore prefer to leave a discussion of them to a later publication.

Preliminary qualitative experiments have shown that the products of the enzymic splitting of casein are mainly or only peptides. This provides evidence that the enzyme is of the endopeptidase type and not *e.g.* an aminopeptidase. These experiments are being continued to obtain more quantitative information.

A comparison of the properties of this enzyme with the more purified plant proteases described, shows that it is different from papain in many respects. It does not possess reactive SH-groups necessary for activity, does not clot milk, is inhibited by cysteine, etc. On the other hand, it quite resembles trypsin in most of its properties and also in its pH dependence*. In order to get some rough idea of its activity in the purified state it was compared to a preparation of crystalline trypsin. It appeared that per mg of protein this enzyme is about a quarter as active as crystalline trypsin on casein. As the preparation is certainly not yet pure, this fact shows that this plant protease has an activity of the same order of magnitude as the most powerful enzymes purified from animal sources.

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* See NOTE ADDED IN PROOF.

In view of the fact, that an enzyme of this type has, to our knowledge, not yet been described, we propose the name arvensin for it. We believe that this enzyme is quite different from that demonstrated in green peas by MERGENTIME *et al.*[9, 10] which has a pH optimum of 5.5 acting on casein and is present in the supernatant at pH 4.0. The proteolytic enzyme from yellow pea seeds described by DANIELSSON[10] has a pH optimum between 6.3 and 7.0 depending upon the substrate. Whether it resembles arvensin cannot be decided as other properties have not been described.

It is not yet known how the proteolytic activity in germinating seeds is regulated. According to some workers[10, 11] the proteolytic activity increases during germination while according to others it remains constant[12]. It is clear, however, that the enzyme investigated by us, is present in ungerminated seeds. Studies of its level in germinating seeds will be carried out.

NOTE ADDED IN PROOF

The enzyme is not inhibited by diisopropylphosphofluoridate (10^{-3} *M*), even after preincubation for 20 min at 30°.

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REFERENCES

[1] M. L. ANSON, *J. Gen. Physiol.*, 22 (1938) 79.

[2] J. H. NORTHROP, M. KUNITZ AND R. M. HERRIOTT, *Crystalline Enzymes*, Columbia University Press, New York, 1948, p. 310.

[3] H. W. ROBINSON AND C. G. HOGDEN, *J. Biol. Chem.*, 135 (1940) 727.

[4] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.

[5] C. E. DANIELSSON, *Biochem. J.*, 44 (1949) 387.

[6] I. D. RAACKE, *Biochem. J.*, 66 (1957) 101.

[7] T. TEORELL AND E. STENHAGEN, *Biochem. Z.*, 299 (1938) 416.

[8] G. GOMORI, in S. P. COLOWICK AND N. KAPLAN, *Methods in Enzymology*, Academic Press, Inc., New York, 1955, Vol. I, p. 144.

[9] M. MERGENTIME AND E. H. WIEGAND, *Fruit Products J.*, 26 (1946) 72.

[10] C. E. DANIELSSON, *Acta Chem. Scand.*, 5 (1951) 791.

[11] H. TAUBER AND S. LAUFER, *Federation Proc.*, 2 (1943) 72.

[12] J. L. YOUNG AND J. E. VARNER, *Arch. Biochem. Biophys.*, 84 (1959) 71.

ENERGY EXPENDITURE BY ACTIVE TRANSPORT MECHANISMS

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SUMMARY

The energy expended by an active transport process has been derived from the physico-chemical features of a hypothetical transport mechanism on the basis of a general "Carrier" model. For this purpose the transport system proper has been narrowly defined as including only those reactions in which the transported particle, or carrier, or both, directly participate. The total amount expended can be divided into three fractions, one of them being reversible and the other two irreversible. The reversible energy corresponds to the concentration work actually achieved. The irreversible fractions are connected with the transporting of the transported particle and with the continuous supply of active carrier, respectively. Of the last two only the first one is accessible to experimental measurement with the help of the unidirectional, carrier-bound fluxes of the transported particle. However, as the concentration of the transported particle approaches zero, the second irreversible energy loss also approaches zero and thus the energy expended by the transport process may be found. From various observations with the system of glycine transport into Ehrlich carcinoma cells, some general conclusions concerning the second irreversible fraction and the coupling between transport and metabolism have been tentatively drawn. The data has also been extrapolated to zero concentrations to yield the value of the energy expenditure. Possible mechanisms to account for the value found are then discussed.

INTRODUCTION

An active transport mechanism is able to perform osmotic work at the expense of metabolic energy. Knowledge of the magnitude of this expenditure would help to elucidate the mechanism and its linkage to metabolism. The "reversible" fraction of this energy, *i.e.*, that amount corresponding to the osmotic work actually achieved, can in many cases be estimated with fair accuracy. The total energy expenditure, however, which contains the "irreversible" fraction, *i.e.*, the dissipation indispensably associated with any real work, is hardly known for any system. Many attempts have been made to derive it from the increment of O_2 consumption by a transporting tissue due to an increment of transport activity. Such procedures, however, suffer from serious uncertainties, partly introduced by the dubious assumptions that the basic metabolic rate of the tissue is the same in the resting and the working state and that none of the resting state energy is used by the transport system. Furthermore, the increment of energy consumption also includes energy dissipation by such reactions as phosphorylation, etc., which may be essentially linked to the transport process but cannot be considered an integral part of the mechanism proper. Highly conflicting results, *e.g.* those concerning the efficiency of gastric acid formation, seem to illustrate the shortcomings of this procedure[1].

The present paper deals with the estimate of the energy expenditure of a transport process on the basis of the chemical nature of the underlying mechanism. This procedure requires a clear and narrow definition of the system with respect to all of the reactions intrinsically involved. An ideal result would, of course, be possible only if all of these reactions and their free energies were known[2]. It is felt, however, that in the absence of such knowledge a useful estimate might be possible on the basis of a reasonable model, which is general enough to fit much of the experimental data available. For most systems of active transport, a simplified "carrier" model—the term "carrier" being used in its widest sense—seems to meet these requirements. A great variety of such models have been suggested by numerous authors, almost all of them having in common the following basic features: (a) the substrate A penetrates the transport region from left to right through interaction with one or more acceptors X; (b) the movement of A against the concentration gradient depends on continuous supply of active X on the left side and continuous inactivation of X on the right side; (c) either this supply of active X or the inactivation of X is energetically linked to an exergonic metabolic reaction. The narrowest definition of such a system in terms of the truly integral reactions concerned would include only those reactions in which at least one of either A or X directly participates.

An early attempt to estimate the minimum energy expenditure of such a system was made by HEINZ[3,4]. It was based on the realization that an indispensible amount of free energy is lost while the substrate carrier complex moves along its gradient within the transport region, and that the greater the rate of net transport the greater this gradient is. Under various simplifying assumptions (such as that the complex AX is virtually at equilibrium at both sides of the region and that A cannot pass the membrane except in combination with X) the energy loss per mole of transported substrate (ΔG_{irr}) was expressed by

$$\Delta G_{\mathrm{irr}} = \mathrm{RT} \ln \frac{\text{forward flux}}{\text{forward flux} - \text{net flux}}$$

where R and T are the Gas constant and the absolute Temperature, respectively, ln is the natural logarithm, and the term "forward flux" represents the unidirectional flux of A in the forward direction. The ratio is identical with the flux ratio. It can be shown that the assumption of virtual equilibrium between AX and A and X in the interfaces is not essential for the above formula to be valid. If, however, the membrane is to an appreciable extent permeable to free A in the uncombined form, the flux ratio has to be restricted to the "carrier-bound" fluxes, *i.e.*, to the fluxes of AX only.

A similar formula was used by ZERAHN[5] in order to estimate the energy necessary to overcome the "internal resistance" of the Na-pump in frog skin. However, no specification was made with respect to "carrier bound" and free diffusion fluxes.

These formula, though representing indisputable losses of energy and though applicable to experimentally accessible data, are deficient in one important respect. Since they consider only reactions in which A directly participates, and ignore reactions with X alone, they are based on too narrow a definition of the transport mechanism. Hence, they give an incomplete account of the energy budget, and the value they provide may fall considerably short of the real energy dissipation unless the substrate concentrations are vanishingly small[2].

In the following, the problem is approached in a more general way in order to assess the error involved in using the above formula. This approach is based on the view that both substrate and carrier are integral parts of the transport mechanism. It will be shown that the estimate as found by the above formula will always be less than the true value of the expended energy but will always be a better estimate than that found by simply taking the maximum concentration ratio that the system as a whole exhibits in a series of experiments.

DERIVATION

In the following derivation the carrier model for active transport will be assumed. The carrier is assumed to operate within a membrane which separates two homogeneous and ideally mixed fluid compartments on the left (l) and on the right (r). Across this membrane a single electrically neutral particle, A, is actively transported from compartment l to r with the help of a carrier X which is able to combine with a single A to form a complex and which is continuously supplied at the interface l, *i.e.*, between the membrane and compartment l. This complex is able to move across the membrane to interface r either by diffusion, rotation, rearrangement of the membrane texture, or some other process. At interface r it may exchange its A with other A in compartment r, or it may unload its A. Then through a series of reactions and/or diffusion the carrier will return to interface l. It will be further assumed that all of the reactions involving A or the complex proceed via unitary reactions which obey mass action kinetics and that all of the constituents involved in the reactions are electrically neutral. Since the resultant equation is valid under very general conditions, a general system will be chosen.

Before the A_l combines with the carrier, it may first react with other particles. These reactions may be written as

$$\begin{aligned} A_l + S_1 &\underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} A^{(1)} + P_1 \\ A^{(1)} + S_2 &\underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} A^{(2)} + P_2 \\ &\vdots \\ A^{(n-1)} + S_n &\underset{k_{-n}}{\overset{k_n}{\leftrightarrows}} A^{(n)} + P_n, \end{aligned} \tag{1}$$

where the k_i are the unidirectional rate coefficients, the positive subscripts referring to the forward direction and the negative ones referring to the reverse direction; the $A^{(i)}$ are intermediates containing the A; and the S's and P's represent substrates and products respectively with all or none of the S's and P's present in an actual system. (The S's and P's may represent enzymes, or energy yielding reactants which "activate" the A, such as ATP and ADP respectively.)

The next step would be for the $A^{(n)}$ to combine with the X_l at the left interface and by a series of reactions and/or diffusion, X_r will appear at the right interface and the A will be liberated, perhaps in a form different from its free form. (Note that diffusion, under the assumption of no interaction between fluxes, may be represented by a first order reaction in which the forward and backward rate coefficients are the same and are equal to the permeability constant.) This series of reactions may be represented as

$$\begin{gathered}
A^{(n)} + X_l + S_{n+1} \underset{k_{-(n+1)}}{\overset{k_{n+1}}{\rightleftarrows}} A^{(n+1)} + P_{n+1} \\
A^{(n+1)} + S_{n+2} \underset{k_{-(n+2)}}{\overset{k_{n+2}}{\rightleftarrows}} A^{(n+2)} + P_{n+2} \\
\vdots \\
A^{(m-1)} + S_m \underset{k_{-m}}{\overset{k_m}{\rightleftarrows}} A^{(m)} + P_m + X_r
\end{gathered} \tag{2}$$

The $A^{(n+1)}, \ldots, A^{(m-1)}$ will all be complexes of the A, the X and perhaps other particles.

Finally, the $A^{(m)}$ will react with other substrates and/or enzymes until it becomes a free particle. These reactions are

$$\begin{gathered}
A^{(m)} + S_{m+1} \underset{k_{-(m+1)}}{\overset{k_{m+1}}{\rightleftarrows}} A^{(m+1)} + P_{m+1} \\
\vdots \\
A^{(p-1)} + S_p \underset{k_{-p}}{\overset{k_p}{\rightleftarrows}} A_r + P_p
\end{gathered} \tag{3}$$

The overall reaction for the transport of the A and the carrier from compartment l to compartment r may then be written as

$$A_l + X_l + S_1 + S_2 + \ldots + S_p \rightleftharpoons A_r + X_r + P_1 + P_2 + \ldots + P_p \tag{4}$$

If it is assumed that the X_r will then return to compartment l through only one other pathway than that shown by Eqn. 4, it will go through a series of steps which may be summarized as

$$X_r + S_{p+1} + S_{p+2} + \ldots + S_q \rightleftharpoons X_l + P_{p+1} + P_{p+2} + \ldots + P_q \tag{5}$$

The overall reaction for the transport of one molecule of A from the left compartment to the right compartment may then be represented as

$$A_l + S_1 + \ldots + S_q \rightleftharpoons A_r + P_1 + \ldots + P_q \tag{6}$$

The free energy change of the overall reaction (Eqn. 6), ΔF_T, may be written as the sum of the ΔF's of the reactions shown in Eqns. 4 and 5.

Therefore

$$\Delta F_T = \Delta F_A + \Delta F_X, \tag{7}$$

where

$$\Delta F_A = \mathrm{RT}\ \ln \frac{A_r X_r P_1 \ldots P_p}{A_l X_l S_1 \ldots S_p} + \sum_{i=1}^{p} \mu_{0P_i} - \sum_{i=1}^{p} \mu_{0S_i} + \mu_{0X_r} - \mu_{0X_l}, \tag{8}$$

and

$$\Delta F_X = \mathrm{RT} \ln \frac{X_l P_{p+1} \ldots P_q}{X_r S_{p+1} \ldots S_q} + \sum_{i=p+1}^{q} \mu_{0P_i} - \sum_{i=p+1}^{q} \mu_{0S_i} + \mu_{0X_l} - \mu_{0X_r}, \tag{9}$$

where μ_0 is the standard chemical potential, the symbols A, X, S, and P represent concentrations, as well as representing the particle itself, and it is assumed that $\mu_{0A_l} = \mu_{0A_r}$. Since the sum of the standard chemical potentials of Eqn. 8 is equal to minus RT times the log of the equilibrium constant in the reaction of Eqn. 4, which in turn is equal to minus RT times the log of the product of the forward reaction rates, divided by the product of the backward reaction rates, Eqn. 8 may be rewritten as

$$\Delta F_A = \mathrm{RT} \ln \frac{A_r X_r P_1 \ldots P_p\, k_{-1} \ldots k_{-p}}{A_l X_l S_1 \ldots S_p\, k_1 \ldots k_p}. \tag{10}$$

If the pump is in the steady state, and if $\overrightarrow{\mathrm{J}}$ and $\overleftarrow{\mathrm{J}}$ are the unidirectional carrier fluxes of A_l towards the right compartment and A_r towards the left compartment respectively, then it can be easily shown[6], that for the reactions as shown in Eqns. 1, 2, and 3,

$$\frac{\overleftarrow{\mathrm{J}}}{\overrightarrow{\mathrm{J}}} = \frac{A_r X_r P_1 \ldots P_p\, k_{-1} \ldots k_{-p}}{A_l X_l S_1 \ldots S_p\, k_1 \ldots kv_p}. \tag{11}$$

It can be seen from Eqn. 6, that the total energy, W, put into the system in order to transport one mole of A from compartment l to r is, as discussed in ref. 2,

$$W = \mathrm{RT} \ln \frac{S_1 \ldots S_q}{P_1 \ldots P_q} + \sum_{i=1}^{q} \mu_{0S_i} - \sum_{i=1}^{q} \mu_{0P_i}. \tag{12}$$

It should be emphasized that W represents the energy expended by the reactions which are directly coupled to the active transport mechanism and not the energy expended by reactions which are only secondarily involved. For example, if the S's and P's represented ATP and its products, and if the ATP was regenerated by creatine phosphate breakdown, then the W would represent only the ATP reaction and not the creatine phosphate reaction. Thus, if the O_2 consumption method actually measured the O_2 required for the transport mechanism, it would yield an energy consumption which is greater than or equal to W since some of the O_2 might be involved in reactions which generate the S's.

The result of combining Eqns. 7 through 12 is then

$$W = \mathrm{RT} \ln \frac{A_r}{A_l} + \mathrm{RT} \ln \frac{\overrightarrow{\mathrm{J}}}{\overleftarrow{\mathrm{J}}} - \Delta F_X. \tag{13}$$

DISCUSSION

Eqn. 13 shows that the total energy fed into the transport system, as defined at the outset, consists of three fractions. The fraction represented by the term RT ln (A_r/A_l) is the osmotic work actually achieved by the system. The other two fractions, which are equal to $-\Delta F_T$, give the waste of energy, *i.e.*, the amount of energy irreversibly lost through the mechanism. The first of these terms, RT ln $(\overrightarrow{J}/\overleftarrow{J})$, or $-\Delta F_A$, covers only those reactions of the systems in which the substrate A directly participates. It is, as such, numerically identical with ΔG_{irr} in the early formula proposed by HEINZ[3,4] to represent the minimum loss of energy associated with any real work of the system. The last term, $-\Delta F_X$, gives the energy wasted by the series of processes involved in reactivating X at the expense of metabolic energy. This term, though referring to an integral part of the transport system proper, is, contrary to $-\Delta F_A$, not accessible experimentally.

We will now further assume that the concentrations of A and the orientation of the active transport mechanism are such that there is a net active transport from left to right, *i.e.*, $\overrightarrow{J} \geq \overleftarrow{J}$. Thus, the reactions of Eqns. 4 and 5 proceed from left to right, and therefore $\Delta F_X \leq 0$. Hence,

$$\text{RT} \ln \frac{A_r}{A_l} \leq \text{RT} \ln \frac{A_r}{A_l} + \text{RT} \ln \frac{\overrightarrow{J}}{\overleftarrow{J}} \leq W \cdot \tag{14}$$

As shown previously[2], under the above assumptions, (in particular that the X traverses the membrane via only two pathways, one with and one without the A), as the concentration of the A's approach zero, the inequality sign on the right side of Eqn. 14 may be replaced by the equality sign. That is, as the A's approach zero, the fluxes approach zero, the reaction of Eqn. 5 approaches equilibrium, and therefore ΔF_X approaches zero.

Eqns. 13 and 14 were derived under the following implicit assumptions: (a) The activity coefficient of the A's is unity. Otherwise the equations are valid only if A is considered to represent activities, since reaction kinetics actually involve activities and not concentrations alone[7]. (b) The standard chemical potential, μ_0, of A_l and A_r are identical. Otherwise the partition coefficient of the A's between the left and right compartments must be introduced into Eqns. 13 and 14.

One of our assumptions was that the l and r refer to the regions immediately adjacent to the carrier. However, the carrier may only operate within a subregion of the total membrane, as in gastric mucosa, frog skin, etc. Thus, l and r may not be the left and right solutions on either side of the membrane. If the A's now represent the activities times the partition coefficient, the ratio of the A's in the right and left solution will, under the assumptions listed above, be less than or greater than the ratio for the A's on either side of the carrier, dependent on whether the net flow (passive plus active) is from left to right or from right to left respectively. If the system is in pseudo-equilibrium, *i.e.*, the net flow is zero[8], or if the permeability of the regions on either side of the membrane is very large, then the ratios for the two regions will be equal and the l and r may refer to the left and right solutions on either side of the membrane.

W need not be a constant for any given system since the concentrations of the

S's and the P's may change during the course of an experiment. If there is an enzyme, it will appear in both the numerator and denominator of Eqn. 11 and will cancel out. Thus, variations in the concentrations of enzymes involved in the transport process will not alter the value of W. If there is a sufficient concentration of energy yielding substrates in the experimental environment, it is quite possible that the W may have a constant value independent of the rate of the pump or the concentration of A_l and A_r.

Although the carrier model for active transport was assumed, the same conclusions reached above are applicable for any individual type of active transport mechanism[8].

APPLICATION

The formulas derived in the foregoing section will now be applied to previously published experimental data concerning the accumulation of glycine in EMAT cells[9]. Here the relationship between the fluxes and the transport mechanism for glycine are fairly well understood[10]. Table I, which is derived from data given in the original paper, shows the accumulation ratios, the unidirectional carrier-bound fluxes, the osmotic work, and ΔF_A calculated via Eqns. 10 and 11. If W, the work put into the system in order to transport one mole of A, is constant and independent of the concentration of A_l and A_r, then from Table I and Eqn. 13 we have to conclude that the system becomes less efficient with increasing A_l. Hence, either ΔF_A, ΔF_X, or both, have to increase in order to compensate for the decrease in A_r/A_l. Since ΔF_A, as derived from the experimental flux ratio (Table I), does not increase with the substrate concentration, the loss of efficiency has to be attributed to an increase of ΔF_X only. Such an increase must result from an increase of the ratio X_r/X_l. It can well be conceived that X_l decreases with an increase of A_l, but an increase of X_r/X_l would imply that the concomitant decrease of X_r is not as pronounced. It is interesting to recall that the carrier-bound efflux of glycine rises proportionately with A_r, *i.e.*, that the carrier-bound efflux coefficient h is independent of A_r over a wide range of values[9]. Hence, the carrier-bound efflux, being the reverse reaction

TABLE I

CONCENTRATION WORK (RT ln A_r/A_l) AND ΔF_A AT VARIOUS EXTRACELLULAR GLYCINE CONCENTRATIONS AND UNDER PSEUDO-EQUILIBRIUM DISTRIBUTIONS

The data given is derived from that presented in a previous paper[9]. The "carrier-bound" fluxes have been obtained by subtracting the contribution of free diffusion (leakage) from the total unidirectional fluxes of glycine. T has the value of 310° K.

Extracellular glycine (A_l) *mmoles*	*Pseudo-equilibrium distribution ratio of glycine* (A_r/A_l)	*"Carrier-bound" fluxes*		*Osmotic work* (RT ln A_r/A_l) *cal/mole*	ΔF_A (RT ln $\vec{J}/\overleftarrow{J}$) *cal/mole*	$W - \lvert \Delta F_x \rvert$ (RT ln A_r/A_l + RT ln $\vec{J}/\overleftarrow{J}$) *cal/mole*
		Influx ($\vec{J}$) *µmole/g · min*	*Efflux* ($\overleftarrow{J}$) *µmole/g · min*			
1.7	14.4	23.8	19.2	1645	129	1774
1.7	12.0	19.6	15.8	1528	129	1657
6.9	6.9	48.1	40.0	1189	111	1300
7.6	6.6	51.6	43.0	1164	111	1275
16.5	4.5	73.4	61.7	924	105	1029
16.5	5.0	82.2	69.0	992	105	1097
31.4	2.5	77.4	68.0	567	80	647
31.4	2.4	76.5	67.5	542	74	616

of Eqn. 4, is determined by A_r. This seems to indicate that under such conditions X_r is fairly constant, even at higher substrate concentrations, which is in agreement with the comments above.

To obtain W, according to Eqn. 13, ΔF_X has to be eliminated by extrapolating the experimental values to $A_l = A_r = 0$. Since both fluxes, $\overrightarrow{J}$ and $\overleftarrow{J}$, are required, Eqn. 1 of the paper concerned[9], has to be rewritten. We shall assume that the X influx through the carrier, $\overrightarrow{J}$, may be represented by a Michaelis-Menten relationship and that the outflux through the carrier, $\overleftarrow{J}$, is linear with A_r. Thus, if A_r and A_l represent the intracellular and extracellular concentrations of glycine, respectively, corresponding to a_c and a_f in the previous paper; K_L is the "leakage coefficient" (diffusion across cell membrane) determined to be about 0.2 ml/g·min; K_m is a figure analogous to the Michaelis constant; $\overrightarrow{J}_{\text{max}}$ is the maximum carrier flux; and h is the coefficient of the carrier-bound efflux, found to be independent of A_l over a wide range of values of A_r and to be about 0.8 ml/g·min[10]; then in pseudo-equilibrium the above assumptions lead to

$$K_L(A_r - A_l) = \frac{\overrightarrow{J}_{\text{max}} A_l}{K_m + A_l} - hA_r. \tag{15}$$

As A_l approaches zero, $\overrightarrow{J}$ will approach the limiting value $(\overrightarrow{J}_{\text{max}}/K_m)\, A_l$ and $\overleftarrow{J}$ will be $h\,A_r$. Inserting these values into Eqn. 13, and using the fact that ΔF_X vanishes as A_l and A_r approach zero, we find that

$$W = \text{RT} \ln \frac{\overrightarrow{J}_{\text{max}}}{K_m\, h}. \tag{16}$$

In order to find $\overrightarrow{J}_{\text{max}}/K_m$, Eqn. 15 may be rearranged to yield

$$\frac{5\,A_l}{5\,A_r - A_l} = \frac{A_l}{\overrightarrow{J}_{\text{max}}} + \frac{K_m}{\overrightarrow{J}_{\text{max}}}. \tag{17}$$

We obtain a straight line by plotting $5A_l/(5A_r - A_l)$ versus A_l with the data derived from Fig. 3 of ref. 9 (Fig. 1). The intercept yields a value of $\overrightarrow{J}_{\text{max}}/K_m = 16.8$ ml/g·min and we find from the slope that $\overrightarrow{J}_{\text{max}}$ has a value of 73.7 μmoles/g·min. As a check on the method, a slightly different approach may be used. Letting $\overrightarrow{J}$ equal the first term on the right side of Eqn. 15, we have, after elementary rearrangement,

$$\frac{A_l}{\overrightarrow{J}} = \frac{A_l}{\overrightarrow{J}_{\text{max}}} + \frac{K_m}{\overrightarrow{J}_{\text{max}}}. \tag{18}$$

We obtain a straight line by plotting $A_l/\overrightarrow{J}$ versus A_l with the data from Table I, (Fig. 2). The intercept of this line with the ordinate yields a value of $\overrightarrow{J}_{\text{max}}/K_m$ of 17.6 ml/g·min while the slope yields a value for $\overrightarrow{J}_{\text{max}}$ of 92.6 μmoles/g·min. The two approaches yield results which agree as well as could be expected. Taking $\overrightarrow{J}_{\text{max}}/K_m$

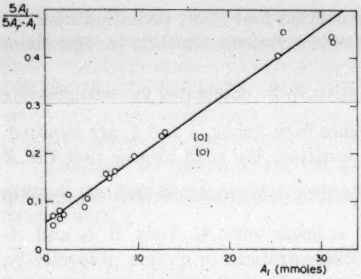

Fig. 1. Steady state distribution of glycine between intracellular fluid and medium at various extracellular concentrations, plotted according to Eqn. 17. The values are derived from those presented in Fig. 3 of a previous publication[9]. From the intercept of the regression line which has been obtained by the method of least squares, $K_m/\overrightarrow{J}_{max}$ can be computed to be about 16.8 ml/g·min.

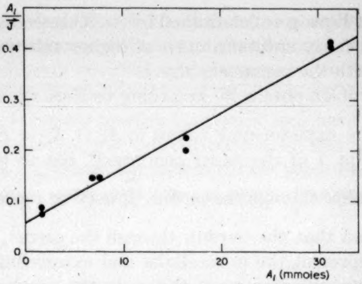

Fig. 2. Relationship between extracellular glycine concentration (A_i) and influx ($\overrightarrow{J}$) during the steady state at various levels of A_i plotted according to Eqn. 18. The values are derived from those presented in Table I of this paper and originally published in a previous paper[9]. From the intercept of the regression line, which has been obtained by the method of least squares, $K_m/\overrightarrow{J}_{max}$ can be computed to be about 17.6 ml/g·min.

to be 17.2 ml/g·min and h to be 0.8 ml/g·min, and inserting these values into Eqn. 16, we find that $W = 1{,}890$ cal/mole. In order to see how W varies as the above parameters are changed, we may consider some plausible extremum values. For $\overrightarrow{J}_{max}/K_m = 16$ ml/g·min and $h = 0.9$ ml/g·min, $W = 1{,}768$ cal/mole, while for $\overrightarrow{J}_{max}/K_m = 18$ ml/g·min and $h = 0.7$ ml/g·min, $W = 2{,}000$ cal/mole. Thus $W = 1{,}890 \pm 120$ cal/mole.

As stated before, we have assumed that the A can only cross the membrane in combination with the carrier via a single pathway. Thus, the A cannot also cross the membrane in conjunction with X via a second pathway which is not coupled with the energy yielding reactions. Though this latter possibility cannot be completely excluded by the experimental data, it may be considered to be a comparatively small effect if it exists at all. We have also assumed above that the influx is given by a Michaelis-Menten reaction and is independent of the intracellular concentration. Actually, the latter assumption is false as can be seen from Fig. 3 of ref. 10. However, since the fit of Eqns. 17 and 19 to the data is fairly good, since we are considering small values of A_i, and since in the limit the effect of the intracellular concentration on $\overrightarrow{J}$ should be negligible, this fact may not be too important. However, further experiments are needed. If the above effect of the intracellular concentration is non-negligible, then the values of $\overrightarrow{J}$ are larger than they would be for $A_r = 0$. Thus, the value of $\overrightarrow{J}_{max}/K_m$ would then be smaller than was actually found from Figs. 1 and 2, and hence the value of W would also be smaller.

The value of $W = 1{,}890$ cal/mole represents the free energy of reaction 6, which has been considered constant and independent of the transport rate. This figure gives the first reasonable estimate so far obtained for any real transport system on the basis of experimental data. Some general conclusions as to the special features of the transport mechanism may now be drawn.

Evidence has been reported previously that energy-rich phosphates are most likely essential for the energy supply in this transport system[12]. This has also been postulated for almost every other system. The magnitude of W, however, seems to rule out that such phosphates interact directly with the transport mechanism. In other words, reaction 6 can hardly be considered as representing the exergonic splitting of ATP or related substances, because in that case W should be of the order of 7,000 to 8,000 cal/mole. It must rather be postulated that the coupling between energy-rich phosphates and the transport mechanism is through one or more energy transferring intermediates. The last of these intermediates would be among the S's of Eqn. 7, where energy-rich phosphates do not appear in this equation. In this connection the question arises as to the stoichiometric ratio of this coupling, *i.e.*, whether one or more glycine molecules are transported per phosphate bond.

The analogy to other reactions coupled with ATP splitting, such as the hexokinase reaction, or the activation of amino acids would suggest a one to one ratio. This would mean, however, that more than 5 kcal were dissipated with each reaction between ATP and S, *i.e.*, before the energy is transferred to the transport mechanism proper. If, on the other hand more than one glycine molecule is transported per phosphate bond, in analogy to the ratio assumed for the Na-transport across the frog skin, two possibilities may be conceived. First, the coupling ratio between phosphate and carrier is one, but more than one glycine molecule can be transported by each carrier. It is difficult to imagine a carrier of this kind in terms of organic chemistry. Also, it should be remembered that the influx and outflux data fit the kinetics of first order reactions. The other possibility would be that the total free energy liberated from the splitting of ATP be proportioned to more than one distinct reaction of lower energy. For this purpose the liberation of inorganic P would have to occur in several steps, each of which is coupled to an endergonic reaction such as activation of P_i to S_i in eqn. 6.

Such a mechanism would be analogous to that of the respiratory chain, where the transfer of one pair of electrons is step-wise coupled to the formation of several energy-rich phosphate bonds. No such step-wise coupling of the splitting of a phosphate bond is hitherto known for any biochemical system. It is therefore highly speculative to postulate this kind of mechanism for active transport. Since the maximum rate of glycine transport in Ehrlich cells is low enough to be compatible with a coupling ratio of one to one, it cannot be decided at the present time whether, and in which way, one or more glycine molecules are transported per phosphate bond.

Another possibility is that the transport of glycine is driven by the potassium ion gradient across the membrane[13,14]. If this were true, W should be equal to the osmotic work value of the potassium ion distribution. Assuming that the concentration of internal potassium is 134 mM, the concentration of external potassium is 4 mM, and that the electric potential across the membrane[15] is —15 mV, the value of the osmotic work is then 1810 cal/mole which is in excellent agreement with the value of 1890 ± 120 cal/mole found above.

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REFERENCES

[1] E. HEINZ AND K. J. ÖBRINK, *Physiol. Rev.*, 34 (1954) 643.
[2] C. PATLAK, *Bull. Math. Biophysics*, 19 (1957) 209.
[3] E. HEINZ, *Proc. Meeting on Gastroelectrical Phenomena*, New York, 1954, p. 9.
[4] E. HEINZ, *Klinische Wochenschrift*, 34 (1956) 419.
[5] K. ZERAHN, *Acta Physiol. Scand.*, 36 (1956) 300.
[6] J. Z. HEARON, *Physiol. Rev.*, 32 (1952) 499.
[7] S. GLASSTONE, K. J. LAIDLER AND H. EYRING, *The Theory of Rate Processes*, New York, 1941.
[8] C. PATLAK, *Bull. Math. Biophysics*, 18 (1956) 271.
[9] E. HEINZ AND H. A. MARIANI, *J. Biol. Chem.*, 228 (1957) 97.
[10] E. HEINZ AND P. M. WALSH, *J. Biol. Chem.*, 233 (1958) 1488.
[11] H. N. CHRISTENSEN, in W. D. MCELROY AND H. B. GLASS, Ed., Baltimore, *Amino Acid Metabolism*, 1955, p. 63.
[12] E. HEINZ, *J. Biol. Chem.*, 225 (1957) 305.
[13] T. R. RIGGS, L. M. WALKER AND H. N. CHRISTENSEN, *J. Biol. Chem.*, 233 (1958) 1479.
[14] H. N. CHRISTENSEN, *Adv. Protein Chem.*, 15 (1960), in the press.
[15] H. G. HEMPLING, *J. Gen. Physiol.*, 41 (1958) 565.

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EFFECT OF SOME CONTRACTURE-PRODUCING AGENTS ON GLYCEROL-EXTRACTED MUSCLE FIBER RELAXED WITH RELAXING FACTOR

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SUMMARY

1. Caffeine, Ca ion, carnosine, DNP, nicotine and cyanide produced contraction of the fiber relaxed with the crude extract. Acetylcholine, choline, histamine and fluoride had no influence on the relaxed fiber.

2. In the absence of the relaxing factor, caffeine and carnosine had no marked influence on the tension development of the fiber in concentrations ranging from 2 m*M* to 10 m*M*. DNP lowered the tension development to about 50 % of the control value, but cyanide increased it to about 150 % at 10 m*M*. Nicotine increased it to 130 % at 5 m*M* and lowered it slightly at 10 m*M*.

3. These agents had no influence on the fiber relaxed with EDTA except Ca ion and nicotine.

4. Caffeine, DNP and carnosine did not contract the fibers relaxed with crude extract which were preincubated with ATP and Mg. However, Ca ion, nicotine and cyanide constantly produced contraction of the relaxed fibers.

5. On the basis of these findings, the production of relaxing substance by the granules in the presence of ATP and Mg and the significance of the granules in the excitation–contraction coupling were suggested.

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INTRODUCTION

Glycerol-extracted muscle fibers contract on addition of ATP, but this contraction is not followed by relaxation. In the past few years, since MARSH[1] discovered a physiological relaxing factor in an extract of skeletal muscle, many investigations on the nature of the relaxing factor have been carried out[2–12]. It is now generally recognized that the granules, microsomes, isolated from the extract of muscle are essential for the relaxation of the fiber[6–12].

Recently, on the basis of detailed experiments on the inhibiting action of granules on myofibrillar ATPase, NAGAI *et al.*[13] have suggested that a very effective and labile relaxing substance might be produced by granules in the presence of ATP, Mg and oxalate. GERGELY[12] also has reported that the relaxation of fibers may be brought about by some relaxing substance produced by the granules in the presence of ATP and a "co-factor".

It has been known for a long time that many agents, acetylcholine, caffeine, nicotine, calcium, DNP and cyanide, produce contracture of skeletal muscle[14–16]. According to HAYASHI[17], carnosine causes contraction upon intracellular injection. It is interesting to recall at this point the investigation of HASSELBACH[18] in which he observed that the relaxation of glycerol-extracted muscle fiber induced by MARSH–BENDALL factor was inhibited by addition of caffeine, resulting in recontraction of the fiber. It is also well known that the relaxing factor becomes inactive when Ca ions are added. GERGELY[12] reported that carnosine is an inhibitor of the relaxing factor when assayed on both single fiber and myofibrills. Therefore, it can be expected that certain agents which produce contracture of living muscle may show significant effects on the relaxing action of granules in the presence of ATP, Mg and others.

In view of these facts, the present study was carried out for the purpose of throwing some light on the understanding of the nature of the relaxing substance and providing some clue as to the relationship between the excitation and the contraction of muscle.

MATERIALS AND METHODS

The glycerol extraction of muscle fibers from rabbit psoas was carried out according to the method of SZENT-GYÖRGYI[19]. In the present experiment, fibers which had been preserved for 60 days were mainly used. Before the isometric experiment, the above-mentioned muscle bundles were cut and dissected into shorter and fine bundles, about 2 cm long and 0.5 mm in diameter, in a Petri dish containing about 50 ml of 20% glycerol–aqueous solution, and then stored at 0°. After about 12 h, a single fiber was isolated from the above fine muscle bundles under the binocular microscope. The fiber was then mounted on the tensionmeter* as described by WEBER[20]. The diameter of the single fiber was 60–70 μ.

The granules fraction was generally prepared from rabbit muscle according to the procedure of PORTZEHL[9] and BENDALL[21]. The extract was centrifuged at 13,000 × g for 10 min. This separated the large particles of the mitochondria fraction, which were discarded. The supernatant was termed the crude extract. Then this crude

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Abbreviations: ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; DNP, 2,4-dinitrophenol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

extract was spun at 80,000 × *g* for 50 min to precipitate the microsomal particulates, *i.e.*, the relaxing granules. The final residue was resuspended in a solution containing 80 m*M* KCl, 20 m*M* histidine and 5 m*M* potassium oxalate and stored at 0°, and termed the granules fraction.

ATP was obtained from the Sigma Chemical Co. in the form of a crystalline disodium salt. It was dissolved in 20 m*M* aqueous solution which was adjusted to pH 7.0 with KOH before use. Carnosine** used in this experiment was a product of the Nutritional Biochemical Corp. and was only for chemical and investigational use. Other chemicals were obtained from the Kanto Chemical Co. All solutions were prepared with deionized water.

The fibers were tested in solutions containing 0.04 *M* Tris–acetate buffer of pH 7.0, 0.12 *M* KCl, 2 m*M* $MgCl_2$, 2 m*M* ATP and, if desired, the relaxing factor and the contracture-producing agents under investigation. The concentration of the crude extract was expressed in volume per cent of total reaction mixture.

RESULTS

Relaxing activities of the three fractions

The relaxing activities of each of the fractions, *i.e.*, the crude extract, the granules and the supernatant, which had been prepared according to the described method were studied. The fibers developed a maximum tension of about 2400 g/cm² of fiber cross section with 2 m*M* ATP and 2 m*M* Mg at pH 7.0 and room temperature. The granules and supernatant which were obtained from the same volume of the crude extract were far less relaxing than the crude extract (final concentration 10 %). The granules and supernatant recombined in their original proportions were as active as the crude extract, and lowered the tension of the fiber to about 10 % of the maximum tension. These results were in agreement with those of GERGELY[12].

Large amounts of isolated fresh granules corresponding to 60 % crude extract produced relaxation without the addition of supernatant, but became rapidly inactive in a few days. The activity of the crude extract remained for about 7–10 days.

The effect of the contracture-producing agents on the relaxed fibers

At the outset, a single fiber relaxed with crude extract was immersed in the test solution containing caffeine, 2 m*M* ATP, 2 m*M* Mg and 10 % crude extract. As shown in Fig. 1, the relaxed fibers again contracted with increasing concentrations of caffeine. The concentration of caffeine was increased from 0.5 m*M* to 10 m*M*. When relaxation of the fiber was produced with 10 % of crude extract, the second contraction of the fiber appeared on the addition of 1 m*M*, and reached the maximum degree of contraction with 10 m*M* caffeine. The effective concentration of the caffeine changed with variation in the concentration of crude extract. This effect of caffeine was not affected by the addition of 5 m*M* procaine.

Similar results were also obtained in isotonic experiments using fiber bundles, 0.3 mm in diameter.

As can be seen in Fig. 2, Ca ion, nicotine, cyanide, carnosine and DNP showed

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[*] The authors wish to thank Prof. H. H. WEBER, Dr. W. HASSELBACH and Mr. W. HEINEMANN of the Max Planck Institute in Heidelberg for making the tensionmeter.

[**] The authors wish to thank Prof. S. OKAMOTO of the Kobe Medical College for the gift of carnosine.

effects similar to those shown by caffeine. It was also shown that the effects of these agents depended on their concentrations. Ca ion which produced a satisfactory effect in concentrations of about 10^{-4} *M* was most effective as compared with other agents which exhibited their ultimate effects on the fiber relaxed with crude extract in concentrations of 10^{-3} *M*. As illustrated in the figure, the rates of contraction of the fibers in solutions containing the same concentration (5 m*M*) of these agents were in the following order: Ca $\gg$ nicotine $\geq$ caffeine $>$ cyanide $>$ carnosine $>$ DNP. The magnitudes of the maximum tension developed after applying these agents were in the following order: Ca $\gg$ carnosine $>$ cyanide $>$ nicotine $\geq$ caffeine $>$ DNP. In the experiments with caffeine and nicotine, the decreases in tension of the fibers after reaching their maximum tension were exceedingly great compared with any other agents.

In contrast to these agents, acetylcholine, histamine, fluoride and choline had no influence on the relaxation of fibers.

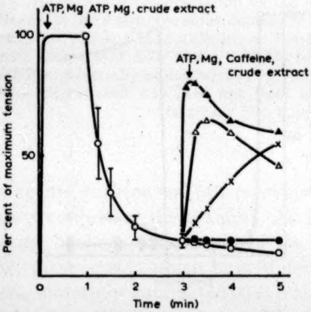

Fig. 1. Effect of caffeine on the fiber relaxed with crude extract. First arrow indicates when fibers were immersed in the test solution containing 2 m*M* ATP, 2 m*M* Mg, 40 m*M* Tris-acetate buffer (pH 7.0) and 0.12 *M* KCl; second arrow, when the ATP solution was replaced with another ATP solution containing 10 vol. % of crude extract and third arrow, replacement with another ATP solution containing 10% of crude extract and caffeine. Caffeine concentration in m*M* is given by ○, nil; ●, 0.5; ×, 2; △, 5; ▲, 10. Room temperature (17–20°).

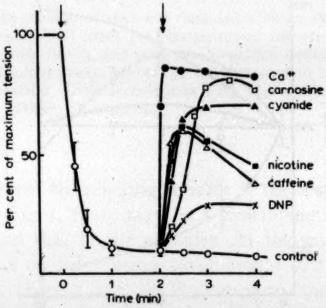

Fig. 2. Effect of several agents on the fiber relaxed with crude extract. First arrow indicates when fibers were immersed in the ATP solution containing 10% of crude extract; second arrow, in the ATP solution containing crude extract and agent. Other conditions were the same as stated for Fig. 1; symbols: ○, nil; ●, 0.2 m*M* Ca; △, 5 m*M* caffeine; ▲, 5 m*M* cyanide; □, 5 m*M* carnosine; ■, 5 m*M* nicotine; ×, 5 m*M* DNP.

The effect of these agents on the tension development

To determine whether the effects of these agents are produced by inhibiting the relaxing factor or by activating the tension development, the experiments shown in Fig. 3 were carried out.

Caffeine and carnosine had no significant influence on the tension development of fibers in concentrations ranging from 2 m*M* to 10 m*M*. The tension development was inhibited with increasing concentrations of DNP and at 10 m*M* the maximum tension was lowered to about 50% of that of the control. Cyanide increased the tension development with increasing concentrations and at 10 m*M* it was increased to about 150% of the control value. Nicotine increased the tension development about 30% at 5 m*M* and lowered it slightly at 10 m*M*.

The effect on the EDTA relaxation

The experiment shown in Fig. 4 demonstrated that no agents except Ca ion and nicotine influenced the fiber relaxed with 2 m*M* EDTA in the presence of 2 m*M* ATP and 2 m*M* Mg.

Influence of preincubation of the crude extract with ATP on the caffeine and DNP effects

Earlier, NAGAI *et al.*[13] presented results showing that the inhibiting activity of granules on the myofibrillar ATPase was increased by preincubation of the granules with ATP, Mg and oxalate. They suggested that the relaxing substance may be produced by the incubation of the granules with ATP.

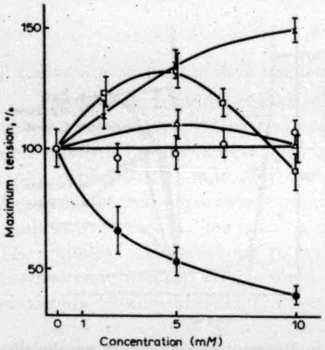

Fig. 3. Effect of several agents on the maximum tension of a single fiber produced with 2 m*M* ATP and 2 m*M* Mg. Ordinate: the maximum tension of fiber developed without agent was taken as 100. Abscissa: concentration of agents in m*M*/l; symbols: ○, caffeine; ●, DNP; ×, cyanide; △, carnosine; □, nicotine.

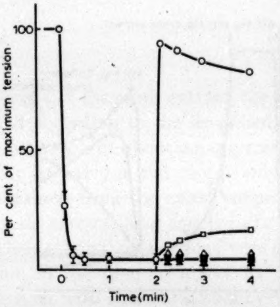

Fig. 4. Effect of agents on the fiber relaxed with EDTA. The relaxation was produced with 2 m*M* EDTA in the presence of 2 m*M* ATP and 2 m*M* Mg. After 2 min the EDTA solution was exchanged with another EDTA solution containing the test agent. Other conditions were the same as stated for Fig. 1; symbols: ○, 2 m*M* Ca; ● 7 m*M* DNP; △, 5 m*M* cyanide; ▲, 5 m*M* carnosine; ×, 5 m*M* caffeine; □, 5 m*M* nicotine.

Accordingly, it would be of interest to know whether the effects of caffeine and the other agents are due to the destruction of the granules or to the inhibition of the production of the relaxing substance.

As shown in Fig. 5, 5 m*M* caffeine and 7 m*M* DNP added to the test solution before the addition of the crude extract produced contraction. However, when 5 m*M* caffeine and 7 m*M* DNP were added to the solution after preincubation of the crude extract with ATP and Mg for 10–30 min, it did not produce contraction of the relaxed fibers. Similar results were obtained with carnosine.

On the other hand, preincubation of the crude extract had no effect on the action of Ca ion, cyanide and nicotine at concentrations of 0.2, 5 and 5 m*M*, respectively. The solutions containing these agents produced contraction of the relaxed fibers, even when each agent was added to the solution after preincubation of the crude extract with ATP and Mg for 30 min*.

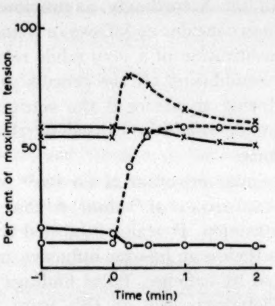

Fig. 5. Influence of preincubation of the crude extract with ATP and Mg on the caffeine and DNP effects. At 0 min, the solution used to decrease the tension of fiber was replaced with another solution to which 5 m*M* caffeine or 7 m*M* DNP was added after preincubation of the crude extract with 2 m*M* ATP and 2 m*M* Mg for 30 min (solid lines), and with one to which caffeine or DNP was initially added without preincubation (broken lines). The crude extract used in the caffeine experiment was used one week after preparation. Other conditions were the same as stated for Fig. 1; symbols: ○, DNP; ×, caffeine.

DISCUSSION

The agents, caffeine and others, evidently inhibited the relaxing activity of the crude extract containing the granules. As shown in Fig. 1, there may be a stoichiometric relation between the amount of the agents and that of the granules. At the same time, these agents which have different effects on the tension development of the fibers, uniformly contracted the relaxed fiber (Figs. 2 and 3). Furthermore, these agents, except Ca ion and nicotine, have no influence on the fiber relaxed with EDTA (Fig. 4). These findings may indicate a specific interaction between these agents and granules. This is further supported by the evidence that the inhibiting activity of granules on the myofibrillar ATPase was removed by the addition of these agents[22].

The results obtained in Fig. 5 indicate that caffeine and DNP do not inhibit the relaxing activity of the crude extract preincubated with ATP and Mg for 10–30 min. Similar results were obtained with carnosine. These findings obtained under the present experimental conditions not only give further weight to the suggestion that a relaxing substance is produced by the granules in the presence of ATP and Mg but also indicate that caffeine and DNP, as well as carnosine, inhibit the chemical reaction which produces the relaxing substance instead of inactivating the substance produced by the granules. On the other hand, Ca ion, nicotine and cyanide inhibited the relaxing activity even when they were added after preincubation of the crude extract for 30 min. Although direct evidence has not yet been obtained, these findings may suggest that these agents might be able to combine with the relaxing substance produced by the preincubation of the crude extract.

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* The authors and Dr. K. Konishi in our laboratory were able to show that 5 m*M* caffeine and 5 m*M* DNP prevent the inhibiting action of granules on the myofibrillar ATPase and that these agents also become less effective on the action of the granules preincubated with ATP, Mg and oxalate for 7 min. Furthermore, they observed that Ca ion, nicotine and cyanide at concentrations of 5 m*M* constantly prevent the inhibiting action of the granules[22].

Furthermore, it is very interesting to note that some of these agents, caffeine[23] and DNP[15], can produce contracture in muscles which are depolarized by application of K_2SO_4 or by removal of Na ion. Accordingly, as previously described by WEBER[24] on the action of Ca ion, one may consider as follows: a living muscle keeps its resting state by maintaining the concentration of a very labile relaxing substance produced by the granules at constant equilibrium. If the process producing the substance is stopped by contracture-producing agents or if the substance combines with these agents, contracture of the muscle would be caused by the decrease in the concentration of the relaxing substance.

It is known that intracellular injection of Ca ion[25] or carnosine[17] causes contraction. On the other hand, AXELSSON *et al.*[23] observed that the intracellular injection of caffeine did not cause contraction. Procaine inhibited the caffeine contracture of frog sartorius muscle[26], nevertheless it has no influence on the contraction of the relaxed glycerol fiber produced by caffeine. These findings appear to contradict the above speculation. Though detailed study on this point is required, the findings obtained in the present work may give a clue to the understanding of the relationship between the excitation and the contraction of muscle and to further investigations for the identification of the relaxing substance.

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REFERENCES

[1] B. B. MARSH, *Nature*, 167 (1951) 1065.
[2] L. LORAND, *Nature*, 172 (1953) 1181.
[3] M. C. GOODALL AND A. G. SZENT-GYÖRGYI, *Nature*, 172 (1953) 84.
[4] J. R. BENDALL, *Proc. Roy. Soc.*, 142 (1954) 409.
[5] H. KUMAGAI, S. EBASHI AND F. TAKEDA, *Nature*, 176 (1955) 166.
[6] C. MOOS AND L. LORAND, *Biochim. Biophys. Acta*, 24 (1957) 467.
[7] H. PORTZEHL, *Biochim. Biophys. Acta*, 24 (1957) 474.
[8] F. N. BRIGGS AND H. PORTZEHL, *Biochim. Biophys. Acta*, 24 (1957) 482.
[9] H. PORTZEHL, *Biochim. Biophys. Acta*, 26 (1957) 373.
[10] S. EBASHI, *Arch. Biochem. Biophys.*, 76 (1958) 410.
[11] H. H. WEBER, *Ann. N.Y. Acad. Sci.*, 81 (1959) 409.
[12] J. GERGELY, *Ann. N.Y. Acad. Sci.*, 81 (1959) 490.
[13] T. NAGAI, M. MAKINOSE AND W. HASSELBACH, to be published.
[14] H. S. GASSER, *Physiol. Revs.*, 10 (1930) 35.
[15] J. M. BARNES AND J. I. DUFF, *J. Physiol.*, 124 (1954) 37.
[16] A. FLECKENSTEIN, *Der Kalium-Natrium-Austausch als Energieprinzip in Muskel und Nerve*, Springer-Verlag, Berlin, 1956.
[17] T. HAYASHI, *The Chemical Physiology of Excitation in Muscle and Nerve*, Nakayama-shioten, 1956.
[18] W. HASSELBACH, in H. H. WEBER, *Biochim. Biophys. Acta*, 12 (1953) 150.
[19] A. SZENT-GYÖRGYI, *Biol. Bull.*, 96 (1949) 140.
[20] A. WEBER, *Biochim. Biophys. Acta*, 7 (1951) 214.
[21] J. R. BENDALL, *Nature*, 181 (1958) 1188.
[22] K. KONISHI, to be published.
[23] J. AXELSSON AND S. THESLEFF, *Acta Physiol. Scand.*, 44 (1958) 55.
[24] H. H. WEBER, *The Motility of Muscle and Cells*, Harvard Univ. Press, 1958.
[25] L. V. HEILBRUNN, *The Dynamics of Living Protoplasma*, Academic Press, New York, 1956.
[26] T. MATSUSHIMA, to be published.

STUDIES ON CARBON DIOXIDE FIXATION BY CHLOROPLASTS

THE EFFECT OF ARSENITE AND OTHER FACTORS

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SUMMARY

A comparison of the rate of photochemical reduction of CO_2 by intact chloroplasts and by fragmented chloroplast preparations was investigated. While arsenite, *p*-CMB, and iodoacetamide inhibited both types of preparations to the same extent, phosphate and arsenate did not.

The site of arsenite inhibition was investigated. The photochemical act was unaffected. None of the enzymes usually associated with the photosynthetic carbon cycle was inhibited.

INTRODUCTION

In a previous paper[1] the effect of pH, classical inhibitors, phosphate, arsenate and other factors on the light induced fixation of CO_2 by intact spinach chloroplasts was reported. In this paper some further results of our experiments on CO_2 fixation by intact and fragmented spinach chloroplasts are described. The most significant finding is the observation that the site of arsenite inhibition in photosynthesis is apparently in the carbon cycle. However, none of the enzymes generally considered to compose the photosynthetic carbon cycle was found inhibited by arsenite. A preliminary report on part of this investigation has appeared[2].

MATERIALS AND METHODS

Preparation of the intact chloroplasts and the reconstituted system

Intact chloroplasts were prepared by methods described in the preceding article[1]. The chlorophyll content of each preparation was determined by the method of ARNON[3] and aliquots containing 1 mg chlorophyll were used in each reaction vessel.

The reconstituted system which consisted of chloroplast extract and fragmented chloroplasts was prepared in the following way. Chloroplast extract was prepared by suspending once washed chloroplasts from 120 g of spinach leaf blades in 9 ml

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Abbreviations: Tris, tris(hydroxymethyl)aminomethane; DPN, TPN, di- and triphosphopyridine nucleotide respectively; FMN, flavine mononucleotide; ADP, ATP, adenosine di- and triphosphate respectively; *p*-CMB, *p*-chloromercuribenzoate, BAL, 2,3-dimercaptopropanol; IAA, iodoacetamide.

of 0.02 M Tris buffer pH 7.5. After 30 min the supernatant fluid was obtained by centrifuging off the chloroplast fragments at 18,000 × g for 20 min.

Chloroplast fragments were prepared by suspending once washed intact chloroplasts from 40 g of leaves in 4 ml of 0.02 M Tris buffer pH 7.5. This suspension was used without further treatment.

Each reaction vessel received chloroplast extract equivalent to 2 mg chlorophyll and fragments equivalent to 1 mg chlorophyll.

Reagents

TPN, FMN, phenazine methosulfate, ADP, ATP, *p*-CMB and phosphoenolpyruvate were products of the Sigma Chemical Company. BAL was obtained from the K and K laboratories. IAA was purchased from the Nutritional Biochemical Company. Solutions of sodium arsenite were prepared from arsenious trioxide (Mallinckrodt Chemical Works) and sodium hydroxide.

Uptake of carbon dioxide

The method used in this investigation was similar to that employed previously[1,4].

Measurement of enzyme activity

Ribulose diphosphate carboxylase, transketolase, transaldolase, neutral and alkaline fructose 1,6-diphosphatases, phosphoriboisomerase, phosphoribulokinase, TPN and DPN associated glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglyceric acid kinase, photoreduction of TPN accompanied by O_2 evolution and photosynthetic phosphorylation activities were assayed by methods employed in previous studies[1,4].

The enzyme, phosphoenolpyruvate carboxylase was assayed by pipetting into a 12 ml centrifuge tube: 0.2 ml of chloroplast extract containing 40 μmoles Tris buffer, pH 7.5, 5 μmoles $MgCl_2$, 1 μmole phosphoenolpyruvate and 5 μmoles of $NaHCO_3$ containing ^{14}C. The total volume was 1.0 ml. After the reaction mixture was allowed to stand at room temperature for 10 min, it was placed in a boiling water bath for 2 min. For measurement of the total tracer fixed, 0.1-ml duplicate aliquots of the contents were pipetted onto aluminum planchets and evaporated to dryness under an infrared lamp. The dry samples were assayed with a gas-flow thin window counter.

For inhibition studies, vessels were kept at 0° until all components had been added. Then inhibitor was permitted to incubate at room temperature for 10 min before the introduction of substrate. When prevention of an inhibition was studied, inhibitor was added after 10 min of incubation, while for reversal studies inhibitor was added at zero time and the test compound was added 10 min later.

RESULTS

pH dependence of CO_2 fixation

Fig. 1 shows the effect of pH on CO_2 fixation by chloroplast fragments supplemented with chloroplast extract. The isotope incorporation rate proceeds at maximal velocity in the vicinity of pH 7.2 to 7.5. Below and above the values, the fixation rate falls off sharply. This result is somewhat similar to that observed with intact chloroplasts[1].

Action of iodoacetamide and p-*CMB on* CO_2 *fixation*

From the results outlined in Table I, it is evident that the alkylating agent, IAA and the mercaptide-forming compound, *p*-CMB, are both potent inhibitors of CO_2 fixation by the reconstituted system. Similar values were obtained previously with the intact chloroplast[1].

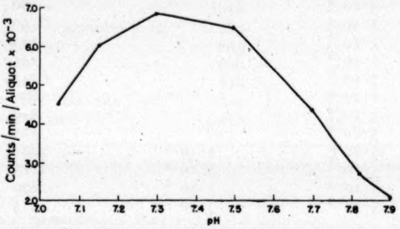

Fig. 1. The effect of pH on CO_2 fixation by the reconstituted system.

TABLE I

EFFECT OF IAA AND *p*-CMB ON $^{14}CO_2$ FIXATION BY THE RECONSTITUTED SYSTEM

Inhibitor concn. (M)	$^{14}CO_2$ *fixed counts/min/aliquot*	% *inhibition*
0	565	—
$5 \cdot 10^{-6}$ IAA	560	1
$1 \cdot 10^{-5}$ IAA	525	7
$5 \cdot 10^{-5}$ IAA	240	58
$5 \cdot 10^{-4}$ IAA	5	99
0	1920	—
$5 \cdot 10^{-6}$ *p*-CMB	1055	45
$1 \cdot 10^{-5}$ *p*-CMB	740	61
$5 \cdot 10^{-5}$ *p*-CMB	115	94

Effect of phosphate on CO_2 *fixation*

Increasing the concentration of phosphate from $5 \cdot 10^{-6}$ *M* to 10^{-2} *M* stimulates CO_2 fixation approx. 14-fold. Higher concentrations inhibit the fragmented chloroplast system. In contrast, the ability of the intact chloroplast to assimilate CO_2 was not stimulated until a concentration of 10^{-3} to $5 \cdot 10^{-4}$ *M* phosphate was reached. In addition, the intact system was strongly inhibited (60 %) by $2 \cdot 10^{-3}$ *M* phosphate[1].

A possible explanation for the effect of phosphate may be found in its action on the enzymes catalyzing the synthesis of ATP or on the enzymes of the reductive pentose phosphate cycle. The increased rate of CO_2 fixation is presumably caused by an accelerated synthesis of ATP (see ref. 5). In contrast, the ribulose diphosphate carboxylase was found to be inhibited by phosphate ions. Thus, in 0.01 *M*, 0.03 *M* and 0.05 *M* phosphate, the activity was only 90, 62 and 42 % of the control value, respectively. WEISSBACH *et al.*[6] have observed the same effect.

With respect to the concentration difference of phosphate in affecting CO_2 fixation in the intact chloroplast in contrast to the fragmented system, no final conclusion can be drawn. The results obtained here do suggest, however, that organization of the enzymes within the chloroplast must surely have a quantitative affect on CO_2 assimilation.

TABLE II

EFFECT OF PHOSPHATE AND ARSENATE ON $^{14}CO_2$ FIXATION BY THE RECONSTITUTED SYSTEM

Phosphate concn. (M)	*Arsenate concn. (M)*	*$^{14}CO_2$ fixed counts/min/aliquot*
0		60
$5 \cdot 10^{-6}$		60
$1 \cdot 10^{-5}$		90
$1 \cdot 10^{-4}$		145
$1 \cdot 10^{-3}$		155
$7 \cdot 10^{-3}$		743
$1 \cdot 10^{-2}$		860
$3 \cdot 10^{-2}$		570
$5 \cdot 10^{-2}$		325
$5 \cdot 10^{-3}$	$5 \cdot 10^{-4}$	2225
$5 \cdot 10^{-3}$	$5 \cdot 10^{-3}$	2115
$5 \cdot 10^{-3}$	$1 \cdot 10^{-2}$	1890
$5 \cdot 10^{-3}$	$3 \cdot 10^{-2}$	315

Table II also reveals that the arsenate ion has an inhibitory effect on CO_2 fixation. This effect is additive to the one caused by phosphate. Like phosphate ions, arsenate ions were inhibitory toward the ribulose diphosphate carboxylase. Thus, 0.03 *M* and 0.05 *M* arsenate inhibited the enzyme 43 and 65 %, respectively. While 0.03 *M* phosphate inhibited the enzyme 38 %, the addition of 0.015 *M* arsenate to the mixture increased inhibition by 12 %.

The reduction of 3-phosphoglyceric acid to 3-phosphoglyceraldehyde catalyzed by 3-phosphoglyceric acid kinase and the TPN-dependent 3-phosphoglyceraldehyde dehydrogenase is also sensitive to arsenate. Thus in 0.02 *M* and 0.05 *M* arsenate ions, the reoxidation of TPNH was only 68 and 26 % of the control value, respectively. The arsenate ions apparently catalyzed an arsenolysis of 1,3-diphosphoglyceric acid.

Effect of arsenite on CO_2 fixation

Arsenite, under the conditions employed, inhibits the CO_2-fixing ability of chloroplast preparations (Table III, IV and V). However, a large variation was found. This variation was in part caused by endogenous material since one wash of

TABLE III

EFFECT OF ARSENITE ON $^{14}CO_2$ FIXATION BY THE RECONSTITUTED SYSTEM PREPARED FROM UNWASHED AND ONCE WASHED CHLOROPLASTS

Condition	*$^{14}CO_2$ fixed counts/min/aliquot*	*% inhibition*
Dark, washed*	80	—
Light, washed	2175	—
Light, washed	730	66
Dark, unwashed**	80	—
Light, unwashed	2460	—
Light, unwashed	1690	31

Arsenite concentration, $5 \cdot 10^{-3}$ *M*

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* Chloroplast fragments and extract were prepared as described in the MATERIALS AND METHODS section.

** Chloroplasts were not washed with 0.35 *M* NaCl before fragments and extract were prepared.

TABLE IV

EFFECT OF BAL ON ARSENITE INHIBITION OF $^{14}CO_2$ BY WASHED WHOLE CHLOROPLASTS

Expt.	*Compounds concn. (M)*	*$^{14}CO_2$ fixed counts/min/aliquot*	*% inhibition**	*% reactivation***
1	Complete	1415	—	—
	BAL $2 \cdot 10^{-4}$	1610	—	—
	BAL $2 \cdot 10^{-4}$, arsenite $7 \cdot 10^{-5}$***	780	52	0
	Arsenite $7 \cdot 10^{-5}$	665	53	—
2	Complete	600	—	—
	BAL $1 \cdot 10^{-3}$	845	—	—
	BAL $1 \cdot 10^{-3}$, arsenite $7 \cdot 10^{-5}$***	220	74	0
	Arsenite $7 \cdot 10^{-5}$	210	65	—

* Percent inhibition = [counts/min (complete) — counts/min (arsenite)]/counts/min (complete) × 100.

** Percent reactivation = [counts/min (after reactivation) — counts/min (arsenite)]/[counts/min (BAL) — counts/min (arsenite)] × 100.

*** Order of addition, BAL at zero time; arsenite 10 min later.

TABLE V

EFFECT OF ARSENITE AND BAL ON $^{14}CO_2$ FIXATION BY THE RECONSTITUTED SYSTEM

Expt.	*Compounds concn. (M)*	*$^{14}CO_2$ fixed counts/min/aliquot*	*% inhibition**	*% reactivation***
1	Complete	3880	—	—
	BAL $5 \cdot 10^{-4}$	1370	65	—
	BAL 10^{-4}	1495	61	—
	BAL $5 \cdot 10^{-4}$, arsenite 10^{-5}***	1035	—	64
	BAL 10^{-4}, arsenite 10^{-5}***	490	—	5
	Arsenite 10^{-5}	440	89	—
2	Complete	630	—	—
	BAL $5 \cdot 10^{-4}$	408	35	—
	BAL $5 \cdot 10^{-4}$, arsenite 10^{-5}***	314	—	75
	Arsenite 10^{-5}, BAL $5 \cdot 10^{-4}$ §	330	—	77
	Arsenite 10^{-5}	35	94	—

* Percent inhibition = [counts/min (complete) — counts/min (arsenite)]/counts/min (complete) × 100.

** Percent reactivation = [counts/min (after reactivation) — counts/min (arsenite)]/[counts/min (BAL) — counts/min (arsenite)] × 100.

*** Order of addition, BAL at zero time; arsenite 10 min later.

§ Order of addition, arsenite at zero time, BAL 10 min later.

the intact chloroplasts resulted in a 2-fold increase in sensitivity to arsenite (Table III).

The data recorded in Table IV and V show that while BAL has little or no effect on the CO_2 fixing ability of the intact chloroplast, it can prevent as well as reverse the inhibition of CO_2 fixation by the reconstituted system caused by arsenite. It is also interesting to note that BAL itself is inhibitory. A similar effect has been noted for pyruvic acid oxidase which is also sensitive to arsenite[7].

Effect of arsenite on individual components of the CO_2-fixing system

Arsenite at 10^{-3} *M*, under the conditions employed, failed to inhibit the following enzymes or systems: ribulose diphosphate carboxylase, transketolase, transaldolase, ribose-5-phosphate isomerase, ribulose-5-phosphate kinase, TPN and DPN glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglyceric acid kinase, phosphoenolpyruvic acid carboxylase, photosynthetic phosphorylation using phenazine methosulfate, vitamin K_3 and riboflavin-5'-phosphate as electron acceptor and photoreduction of TPN accompanied by O_2 evolution and phosphorylation.

DISCUSSION

A comparison of the rate of CO_2 fixation by the intact chloroplast and the reconstituted chloroplast system brought out similarities as well as differences in their behaviour toward certain reagents. Thus, compounds like iodoacetamide, arsenite and *p*-CMB, which are considered to be non-competitive inhibitors affect both systems to the same extent. In contrast, while phosphate and arsenate ions presumably affect the same site in both systems, the concentration of each to attain this effect differs considerably. In addition, the concentrations of intermediates of the various cycles must surely differ in both systems and this may play a role in yielding variation. Clearly, the possibility must be considered that the structural organization plays an important role not only for the photochemical act of photosynthesis but also for subsequent carbon metabolism.

While at least one site of inhibition can be assigned to iodoacetamide, *p*-CMB, arsenate, and phosphate, the particular enzyme, or enzymes affected by arsenite is not clear. The data recorded here on the effect of arsenite on the photolysis of water, the photochemical formation of TPNH and ATP, are in agreement with the results obtained by SAN PIETRO AND LANG[8] and ARNON *et al.*[9] on the synthesis of "reducing power" in broken cell spinach preparations and by MCFADDEN AND ATKINSON[10] on the electron transfer from H_2 to O_2 in the intact, autotrophic bacterium, *Hydrogenomonas facilis*, and are thus consistent with the expectation that the site of arsenite inhibition is apparently the CO_2 reduction cycle.

Most of the enzymes which are usually considered to compose the photosynthetic carbon cycle were tested and found to be insensitive to arsenite.

The susceptibility of an enzyme catalyzed reaction to arsenite has generally been ascribed to the need for lipoic acid as a cofactor[11,12]. More recently, NIRENBERG AND JAKOBY[13] have found that various partially purified aldehyde dehydrogenases are inhibited by arsenite and that lipoic acid is not a participant. In this system, susceptibility is ascribed to the presence of two closely juxtaposed cysteine moieties. In both types, a dimercaptan is able to reverse the inhibition.

In a particulate system, another possibility exists; namely, that two sulfhydryl containing enzymes, individually unreactive toward arsenite, may be brought into proper position to produce a dithiol type structure. On fragmentation, the susceptibility toward arsenite would decrease. However, in the present study, this is apparently ruled out since the fragmented system as well as the intact system yielded similar inhibition values with arsenite.

Clearly, the results of this study does not settle the question of a site of arsenite inhibition in photosynthesis.

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REFERENCES

[1] M. GIBBS AND N. CALO, *Plant Physiol.*, 34, (1959) 318.
[2] M. GIBBS AND N. CALO, *Federation Proc.*, 18 (1959) 235.
[3] D. I. ARNON, *Plant Physiol.*, 24 (1949) 1.
[4] N. CALO AND M. GIBBS, *Z. Naturforsch.*, 15b (1960) 287.
[5] A. T. JAGENDORF AND M. AVRON, *J. Biol. Chem.*, 231 (1958) 277.
[6] A. WEISSBACH, B. L. HORECKER AND J. HURWITZ, *J. Biol. Chem.*, 218, (1956) 795.
[7] O. K. REISS AND L. HELLERMAN, *J. Biol. Chem.*, 231 (1958) 557.
[8] A. SAN PIETRO AND H. M. LANG, *J. Biol. Chem.*, 231 (1958) 211.
[9] D. I. ARNON, F. R. WHATLEY AND M. B. ALLEN, *Biochim. Biophys. Acta*, 32 (1959) 47.
[10] B. A. MCFADDEN AND D. E. ATKINSON, *Arch. Biochem. Biophys.*, 66 (1957) 16.
[11] I. C. GUNSALUS, *Federation Proc.*, 13 (1954) 715.
[12] L. J. REED, *Advances in Enzymology*, 18 (1957) 319.
[13] M. W. NIRENBERG AND W. B. JAKOBY, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 206.

UNBOUND SIALIC ACIDS IN FISH EGGS

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SUMMARY

Relatively large quantities of sialic acids occur in an unbound form in trout eggs.

Both N-glycolyl and N-acetylneuraminic acid are found within one cell, the trout egg.

Preliminary studies on the permeability of the egg to free sialic acids are reported.

INTRODUCTION

Sialic acids[1–3] are found in mammals[4,5], birds, amphibians[5], fish[6,7], bacteria[8], and in the eggs of the sea urchin[9]. Several forms of sialic acids (N-glycolyl, N-acetyl, N,O-diacetylneuraminic acid) have been described in the tissues of different animal species. Virtually all of the sialic acids found in tissues are bound either in large protein-carbohydrate complexes or in oligosaccharides such as neuramin-lactose of the mammary gland[10,11].

In this paper we wish to report some unusual characteristics of the sialic acids of trout eggs. Not only is much of the sialic acid free, but two forms of sialic acid are found in one egg.

MATERIALS AND METHODS

Eggs of the eastern brook trout (*Salzelinus fontinalis*) and rainbow trout (*Salmo gairdneri*) were kindly supplied by Dr. K. E. WOLF, Eastern Fish Disease Laboratory, United States Fish and Wildlife Service, Leetown, West Virginia. The eggs were removed from the female, placed in Tyrode's solution and transported in a container containing crushed ice. Experiments commenced within 20 h of removal from the female and continued for a further 48 h. Eggs were stored in Tyrode's solution at 4°. The eggs of shad (*Alosa sapidissima*) and perch (*Perca flavescens*) were removed from fish obtained from commercial fishermen within 2 h of the time of catching.

Determinations of sialic acids reported in this paper were carried out by the thiobarbituric acid assay[12]. The orcinol assay for sialic acid[13] was used in some of the permeability studies. The thiobarbituric acid assay was carried out on hydrolyzed (80°, 1 h, 0.1 *N* H_2SO_4) (see ref. 14, 15) and unhydrolyzed preparations of egg homogenates. Since the thiobarbituric acid assay measures only unbound sialic acids the two determinations gave the total, free and bound sialic acid content of the eggs. For standards, N-acetylneuraminic acid isolated from blood proteins and N-glycolylneuraminic acid from pig submaxillary gland mucin, were used[16]. The molecular weight of N-acetylneuraminic acid, 309, is used for all calculations.

Ascending chromatography was carried out on Whatman No. 1 paper for 20 h in two solvents which were capable of separating N-acetyl from N-glycolylneuraminic acid. Solvent 1 is *n*-butanol–*n*-propanol–0.1 *N* HCl, (1:2:1) (see ref. 17), Solvent 2, ethanol–water–concentrated ammonia, (80:20:1). Two other solvents were also used: Solvent 3, *sec.*-butanol–acetic acid–water, (4:1:5) and Solvent 4, ethyl acetate–pyridine–water, (1:1:2).

Material isolated from trout eggs was chromatographed alone and also mixed with N-acetylneuraminic acid and N-glycolylneuraminic acid. Sialic acids were located on paper chromatograms by spraying with modified reagents of the thiobarbituric acid assay[18].

RESULTS AND DISCUSSION

It can be seen in Table I that the sialic acid contents of trout, shad and perch eggs are high and that approximately half of the sialic acids of brook and rainbow trout eggs are free. Sialic acids account for over 4% of the dry weight of shad eggs. However,

TABLE I

SIALIC ACID CONTENT OF FISH EGGS

Approximately 1 ml of eggs was added to a graduated tube containing 5 ml of 0.1 *M* KCl solution. The eggs were homogenized in a glass homogenizer and aliquots were used for determination of free and total sialic acid content. Aliquots were also dried *in vacuo* over P_2O_5 for 48 h for dry weight and nitrogen analysis. Determinations were made on 5 samples of brook trout eggs, 3 samples of rainbow trout eggs, and one sample each of shad and perch eggs.

Source	*Free mg/g dry egg*	*Bound* mg/g dry egg*	*Total mg/g dry egg*	*% Free*	*% Nitrogen in dried egg***
Brook trout	2.44 (1.86–2.97)	2.74	5.18 (4.55–5.86)	47.2	11.11
Rainbow trout	8.01 (7.08–8.78)	6.99	15.0 (14.06–16.6)	53.5	10.79
Shad	0.35	43.50	44.10	0.8	11.94
Perch	0.48	21.12	21.60	2.2	8.55

* Calculated by difference.

** Determined by the micro Kjeldahl method.

very little of the sialic acid of shad and perch is free. The free sialic acid of the trout egg was dialysable and could not be precipitated by a solution of 5 % phosphotungstic acid in 2 *N* HCl. Each brook trout egg, with a volume of 0.07 ml, contains a total of 70 μg of sialic acid of which 33 μg is free. In contrast to the large amounts of sialic acid in eggs, the semen of shad and trout contains little or no sialic acids.

In order to see whether sialic acids in eggs were bound in small dialysable oligosaccharides such as neuramin-lactose[10,11], extracts of trout eggs were dialyzed and the dialysis fluid hydrolyzed (0.1 *N* H_2SO_4, 80° for 1 h) and assayed. There was no difference in the values of the sialic acid content of hydrolyzed and unhydrolyzed dialysis fluid as determined by the thiobarbituric acid method. This indicates the absence of small, dialysable sialic acid-containing oligosaccharides.

Characterization of sialic acids

The sialic acids from trout egg resembled authentic sialic acids in that they were adsorbed by Norit A and could be eluted with 50 % aqueous methanol[19]. They also behaved similarly on columns of Dowex-1 formate and Dowex-1 acetate. The absorption spectra in the direct Ehrlich, orcinol, diphenylamine[13], resorcinol[20], and thiobarbituric acid reactions were virtually the same. Neuraminidase, purified from the culture fluid of *Clostridium perfringens*, was capable of freeing the bound sialic acid of trout eggs.

Sialic acids were isolated by chromatographic methods[15,16,21] from hydrolyzed brown trout egg homogenates (total sialic acids), from unhydrolyzed egg homogenates (free sialic acids) and from hydrolyzed egg protein (bound sialic acids). Egg protein was obtained by precipitation, by addition of water to eggs which had been homogenized in physiological saline. In one preparation approximately half of the total sialic acids were found to co-chromatograph in solvents 1 and 2 with N-glycolylneuraminic acid and half with N-acetylneuraminic acid (Table II). The infrared spectrum of this material was identical with that of an equimolar mixture of N-acetyl and N-glycolylneuraminic acid (KBr pellet).

TABLE II

R_F VALUES OF SIALIC ACID FROM TROUT EGGS

Solvent		*Sialic acid from trout ova*		*N-Acetylneuraminic acid*	*N-Glycolylneuraminic acid*
		Free	*Bound**		
1. *n*-butanol	1				
n-propanol	2	0.35 0.45	0.35 0.45	0.45	0.35
0.1 *N* HCl	1				
2. Ethanol	8				
Water	2	0.20 0.30	0.20 0.30	0.30	0.20
Ammonia	1				
3. *Sec*-butanol	4				
Acetic acid	1	0.78	0.76	0.75	0.76
Water	5				
4. Ethyl acetate	1				
Pyridine	1	0.69	0.71	0.71	0.71
Water	2				

* After hydrolysis of egg protein.

Both types of sialic acids were found free and bound and as estimated on paper chromatograms there was great variation from one batch of eggs to another in the relative amount of N-glycolyl to N-acetylneuraminic acid in the free and bound compartments. In one sample no N-acetylneuraminic acid was found in the free form. However, the total amount of sialic acid in several batches of trout eggs was found to be constant.

Permeability studies

Preliminary studies have been carried out on the changes in the concentration of free sialic acid upon fertilization of trout eggs. When trout sperm are added to trout eggs suspended in distilled water at room temperature (23°) there is a marked outpouring of free sialic acid (Table III). However a similar, but variable response has been found upon the addition of boiled sperm, magnesium or calcium ions to eggs suspended in distilled water. Antagonism between calcium and magnesium ions has been observed. Temperature has a marked effect upon the system. Loss of sialic acid does not take place at 0°, but at 37° there is great loss of sialic acid from eggs suspended in distilled water even without additions. It should be emphasized that the system is very sensitive, and is erratic in its response. Whether the loss of sialic acid by eggs has any relevance to the process of fertilization is not known.

TABLE III

RELEASE OF SIALIC ACIDS FROM TROUT EGGS

Each tube contained 4 brown trout eggs in 1 ml of suspending medium which contained the additions listed below. Each egg contains approximately 70 µg of sialic acid of which 33 µg is free. After incubation for 40 min at 23°, 0.75 ml of the medium was removed, 0.75 ml 5% phosphotungstic acid in 2 *N* HCl was added to the aliquot and this was centrifuged. The orcinol reaction[13] was carried out on 1.0 ml of the supernatant solution. Comparable experiments have been carried out in which 0.3 ml of the suspending medium was assayed by the thiobarbituric acid assay without phosphotungstic acid treatment. Concentrations of cations in Expt. B are optimal.

Additions	*µg sialic acid lost per egg*
A. None	2.2
Trout semen	25.2
Trout semen (heated 100°, 15 min)	25.0
Trout semen (no eggs)	0.3
B. None	0.7
$CaCl_2$ (0.1 µmole)	15.0
$MgCl_2$ (1.5 µmoles)	18.0
$CaCl_2$ (0.1 µmole) + $MgCl_2$ (1.5 µmoles)	1.2

DISCUSSION

Free sialic acids have not been described in nature except for small amounts in human cerebrospinal fluid[22]. The amount of free sialic acids in the trout egg is far greater and is a large fraction of the total sialic acid of the cell. Free sialic acid has also been found in hog gastric tissue. However the relatively acidic condition at this site is probably sufficient to cause postmortem release of sialic acid[23]. Sialic acid is known to be bound by acid labile bonds.

The presence of a sialidase (neuraminidase) in trout eggs might account for the occurrence of free sialic acids but none has been detected. Neuraminic acid aldolase[24] also appears to be absent from trout eggs. In this regard there is only 12 % as much free N-acetyl hexosamine as free sialic acid on a molar basis in trout eggs. If neuraminic acid aldolase was present one would expect to find these proportions reversed, for the equilibrium of the aldolase reaction favors degradation of sialic acids.

REFERENCES

[1] G. Blix, *Z. physiol. Chem., Hoppe Seyler's*, 240 (1936) 43.
[2] E. Klenk, H. Faillard and H. Lempfrid, *Z. physiol. Chem., Hoppe Seyler's*, 301 (1955) 235.
[3] A. Gottschalk, *Yale J. Biol. Med.*, 28 (1956) 525.
[4] C. Chatagnon and P. Chatagnon, *Presse méd.*, 63 (1955) 1194.
[5] P. Bohm and L. Baumeister, *Z. physiol. Chem., Hoppe Seyler's*, 305 (1956) 42.
[6] K. Turumi and Y. Saito, *Tokoku J. Exptl. Med.*, 58 (1953) 247.
[7] E. Wessler and I. Werner, *Acta Chem. Scand.*, 11 (1957) 1240.
[8] G. T. Barry, *J. Exptl. Med.*, 107 (1958) 507.
[9] P. Perlmann, H. Bostrom and A. Vestermark, *Exptl. Cell Research*, 17 (1959) 439.
[10] R. E. Trucco and R. Caputto, *J. Biol. Chem.*, 206 (1954) 901.
[11] R. Kuhn and R. Brossmer, *Angew. Chem.*, 68 (1956) 221.
[12] L. Warren, *J. Biol. Chem.*, 234 (1959) 1971.
[13] I. Werner and L. Odin, *Acta Soc. Med. Upsaliensis*, 57 (1952) 230.
[14] L. Svennerholm, *Acta Chem. Scand.*, 12 (1958) 547.
[15] T. Yamakawa and S. Suzuki, *J. Biochem. (Tokyo)*, 42 (1955) 727.
[16] L. Svennerholm, *Acta Soc. Med. Upsaliensis*, 61 (1956) 75.
[17] E. Svennerholm and L. Svennerholm, *Nature*, 181 (1958) 1154.
[18] L. Warren, *Nature*, 186 (1960) 237.
[19] Y. Saito, *Nature*, 178 (1956) 995.
[20] L. Svennerholm, *Biochim. Biophys. Acta*, 24 (1957) 604.
[21] F. Zilliken, G. A. Braun and P. Gyorgy, *Arch. Biochem. Biophys.*, 63 (1956) 394.
[22] L. L. Uzman and M. K. Rumley, *Proc. Soc. Exptl. Biol. Med.*, 93 (1956) 497.
[23] P. Atterfelt, I. Blohme, A. Norby and L. Svennerholm, *Acta Chem. Scand.*, 12 (1958) 359.
[24] D. G. Comb and S. Roseman, *J. Am. Chem. Soc.*, 80 (1958) 497.

Short Communications

Spectrographic determination of molybdenum in the nitrate reductase from *Escherichia coli*

EGAMI *et al.*[1] have separated a nitrate reductase from *E. coli* cells and studied its properties. Some of the results on the solubilization and purification of this enzyme are presented by TANIGUCHI AND ITAGAKI elsewhere. The present authors have collaborated with them in the spectrographic analysis of the metals present in the nitrate reductase and the results obtained are briefly described in this communication.

Qualitative spectrographic analysis of the nitrate reductase samples showed the presence of molybdenum and iron. Molybdenum was determined spectrographically and iron colorimetrically.

For the spectrographic determination of molybdenum in silicate materials, several authors have found that calcium phosphate, alumina and sodium carbonate enhanced the line intensity of molybdenum[2,3], and vanadium and palladium were suitable as internal standards[4,5]. After examination of various substances as base material for the analysis of molybdenum in biological materials, we have found NaCl to be the best, with palladium and vanadium as suitable internal standards[6].

Standard samples of molybdenum containing 5, 10, 26, 52, 104 and 205 p.p.m. MoO_3 which corresponded to 3, 7, 18, 35, 69 and 137 p.p.m. Mo were prepared by thoroughly mixing pure MoO_3 and graphite powder in an agate mortar. These mixtures were then mixed with NaCl and graphite powder containing 50 p.p.m. V and 50 p.p.m. Pd in the ratio of 1:1:1. 8 mg of the standard sample was placed in the hole of the cathode (Fig. 1). A medium quartz spectrograph was used under the following experimental conditions: excitation, D.C. 180 V; cathode layer method; exposure, the current was held at 3, 5 and 7 A. for 30 sec respectively and finally at 9 A until the sample was burnt off; Fuji spectroscopic process plate.

The line pairs selected were Mo 3170.35-V 3185.40 Å and Mo 3170.35-Pd 3442.70 Å. Linearity holds for the former pair in the range 7–140 p.p.m. Mo and for the latter in the range of 35–140 p.p.m. Mo. Therefore vanadium was used as the internal standard and the molybdenum content was calculated by using the working curve shown in Fig. 2.

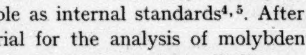

Fig. 1. Electrodes used for the spectroscopic analysis, with cathode below. The dimensions are mm.

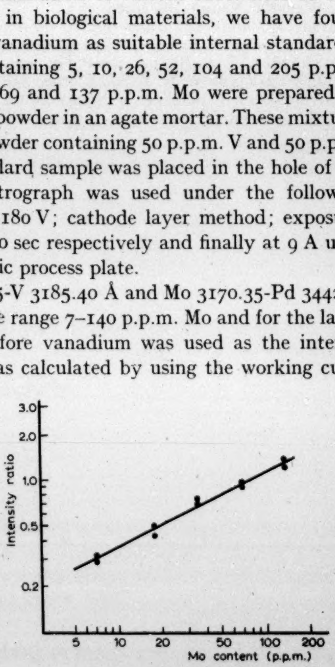

Fig. 2. Standard curve for the spectrographic analysis of molybdenum in the nitrate reductase.

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The nitrate reductase separated from *E. coli* was decomposed by fuming HNO_3 and 30 % H_2O_2 and the residue was mixed with equal amount of graphite powder as described by TANIGUCHI AND ITAGAKI[7]. NaCl and the graphite powder containing 50 p.p.m. V and 50 p.p.m. Pd were then added in the ratio of 1:1:1.

The inactive protein which was separated at an intermediate step of the purification of the nitrate reductase was used as the control material. It was mixed with NaCl and graphite in the same way as the nitrate reductase sample, and other experimental conditions were also the same. The inactive protein contained 11 p.p.m. Mo and this value was used as a correction to the molybdenum content of the nitrate reductase. A dialysate of the nitrate reductase and the inactive protein also contained 11 p.p.m. molybdenum.

Two samples of the nitrate reductase were found to contain 87 p.p.m. and 108 p.p.m. Mo (corrected values). The reproducibility of the spectrographic analysis, measured by analysing repeatedly the inactive protein, was ± 11 % for a molybdenum content of about 11 p.p.m.

A step-sector method was also used to determine molybdenum in the nitrate reductase. In this case both the standard and the nitrate reductase samples were mixed with NaCl in the ratio of 1:1 and excited by the cathode-layer method. A sector with seven steps was placed in front of the slit of the spectrograph and the lengths of the molybdenum lines 3170.35 Å and 3193.97 Å were plotted against the molybdenum contents. A sample of the nitrate reductase was found to contain 96 ± 20 p.p.m. Mo (corrected value) by this method.

Thus the molybdenum contents of three samples of the nitrate reductase were 87, 96 and 108 p.p.m. which corresponds to almost one atom Mo for one molecule of the nitrate reductase.

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[1] F. EGAMI, M. ISHIMOTO AND S. TANIGUCHI, *Proceedings of the Symposium on Haematin Enzymes, Canberra*, 1959, in the press.
[2] R. O. SCOTT, *J. Soc. Chem. Ind.*, 64 (1945) 189.
[3] R. L. MITCHELL, *Commonwealth Bureau of Soil Science, Technical Communication*, 44 (1948) 88.
[4] L. H. AHRENS, *Quantitative Spectrochemical Analysis of Silicates*, Pergamon Press, London, 1954.
[5] T. M. MOROSHKINA AND V. K. PROKOFEV, *Zhur. Anal. Khim.*, 11 (1956) 714; C.A. 51 (1957) 7941f.
[6] C. IIDA, to be published.
[7] S. TANIGUCHI AND E. ITAGAKI, *Biochim. Biophys. Acta*, 44 (1960) 263.

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A method for the determination of free myo-inositol in biological fluids and tissues

Microbiological methods are generally used for the measurement of the myo-inositol content in biological material. More recently, methods have been proposed involving the use of an enzyme prepared from animal tissues[1] or bacteria[2].

During the course of an investigation of the myo-inositol content of adult and fetal fluids and tissues, we have used the chemical method presented in this paper.

It is essentially an adaptation of the general method for sugars described by HIRST AND JONES[3]: myo-inositol is separated from other carbohydrates by paper chromatography, eluted from the chromatogram and oxidized by sodium metaperiodate to yield formic acid, which is then determined titrimetrically.

For routine determinations, the biological material to be analyzed by this method should contain a minimum of 0.3 mg inositiol. The tissues to be analyzed are washed with 0.9 % NaCl at 0° for a few seconds, blotted, placed on solid CO_2 and then weighed while frozen.

Free myo-inositol is extracted from the tissues by grinding in water at room temperature. Proteins are precipitated with $Ba(OH)_2$–$ZnSO_4$ by the method of SOMOGYI[4]. The extract is centrifuged and the supernatant containing free myo-inositol is deionized by passing it through an 0.5 × 6.0 cm mixed-bed ion-exchange resin column (Dowex 50 – X12, H^+; and Dowex 1 – X8, formate$^-$). The eluent is collected in an evaporating dish and dried at 60°. The dry residue is dissolved in water to make up a solution in which inositol has the approximate concentration of 1 μg/μl.

Known amounts of the solution, estimated to contain about 50–400 μg inositol are then chromatographed on 3-cm wide strips of Whatman No. 1 paper by descending chromatography, an extra strip being run for each sample as a marker. The chromatograms are developed with ethyl acetate–pyridine–water (2:1:2). After a 16-h run at room temperature, the strips are dried, the marker strip stained by dipping, with a satd. solution of $AgNO_3$ in acetone and 5 % NaOH in alcohol according to SMITH[5]. By reference to the chromatogram of a standard solution, myo-inositol is easily identified as a slowly developing, dark band close to the origin. Using the stained marker strip as a guide, the area of paper that contains myo-inositol is cut and eluted with 2 ml water. To 1 ml of the eluent, in 10 × 160 mm tubes with screw caps, 0.4 ml sodium meta-periodate (0.25 M) are added. These tubes are placed in a water bath at 100° for 20 min. The tubes are then cooled, 0.2 ml of ethylene glycol are added and the formic acid is titrated.

Rather than titrating the formic acid directly, we found it more convenient to neutralize it with NaOH and titrate the excess NaOH with 0.001 N H_2SO_4 in the presence of phenol red as the indicator. The procedure adopted is the following. In a 50-ml syringe are placed 50 ml aq. 25 % NaCl and to it are added with a micropipette about 17 μl satd. NaOH. In this way a NaOH solution, approx. 0.005 N, relatively free of carbonate even after a brief exposure to air, has been prepared. By way of a stainless-steel three-way stopcock, the 50-ml syringe is attached to a 1-ml Krough-Keyes pipette set to deliver approx. 0.5 ml of solution. With this arrangement it is possible to deliver constant amounts of NaOH, rapidly and precisely, from the 50-ml syringe into the test tubes that contain the formic acid. One or more aliquots of NaOH are delivered until the indicator turns red. The excess NaOH is titrated with the 0.001 N H_2SO_4 from a microburette calibrated to 0.01 ml.

When oxidized by sodium meta-periodate under the conditions described in this method, one mole of myo-inositol yields 4.5 moles of formic acid[3]. As shown in Fig. 1, myo-inositol is fully recovered from the paper after chromatography. The blank due to the paper is on the average 0.2 μequiv. of acid. In a series of 115 duplicate determinations, in which two strips were plated with the same solution and analyzed separately, the average difference in a pair of samples was 0.063 μequiv. of acid, which corresponds to 2.5 μg myo-inositol.

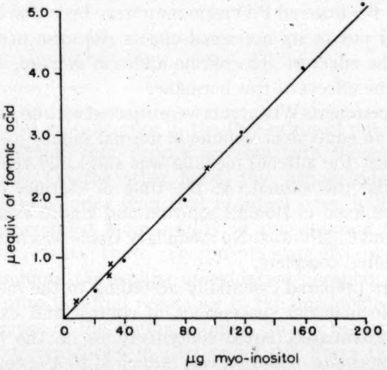

Fig. 1. The diagram represents the relationship between myo-inositol and the formic acid produced by its oxidation. The crosses indicate the results of determinations on known amounts of myo-inositol that were plated on chromatographic paper and then eluted.

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[1] E. L. KEAN AND F. C. CHARALAMPOUS, *Biochim. Biophys. Acta*, 36 (1959) 1.
[2] A. WEISSBACH, *Biochim. Biophys. Acta*, 27 (1958) 608.
[3] E. L. HIRST AND J. K. N. JONES, *J. Chem. Soc.*, (1949) 1659.
[4] M. SOMOGYI, *J. Biol. Chem.*, 160 (1945) 69.
[5] I. SMITH, *Chromatographic Techniques; Clinical and Biochemical Applications*, Heinemann, London, 1958.

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Oxidative phosphorylation in liver mitochondria prepared from adrenal-demedullated and epinephrine-treated rats*

The influence of various hormones upon the efficiency of oxidative phosphorylation has been extensively investigated in recent years. However, studies relating to the effect of the adrenal medullary secretions upon these reactions have not appeared except for the brief report of PARK *et al.*[1]. These investigators observed that addition of epinephrine to suspensions of hamster-liver mitochondria depressed somewhat the P/O ratio obtained with β-hydroxybutyrate as substrate. Substrate levels of epinephrine were employed and since its oxidation yields a P/O ratio of 1 in the cytochrome *c* region[2], the lowered P/O ratio may have been due to electron transport from epinephrine and not to its hormonal effects. Because of this apparent interference in studying the effect of epinephrine addition *in vitro*, a different approach was used to analyze the effects of this hormone.

In one series of experiments Wistar rats were injected with 60 μg L-epinephrine/100 g body weight, or with an equivalent volume of normal saline, 30–45 min before sacrificing. In another series, the adrenal medulla was surgically removed three or more weeks before sacrificing the animal. At the time of sacrifice of the demedullated rats, the adrenals were fixed in Bouin's solution and kindly examined histologically by Drs. J. FROMMER and C. MONROE. No medullary tissue was noted and regeneration of cortical tissue appeared complete.

Mitochondria were prepared essentially according to the method of SCHNEIDER AND HOGEBOOM[3]. Mitochondrial suspensions of control and experimental animals were adjusted to approximately equal concentrations on the basis of absorbancy readings at 510 mμ. Succinate oxidation was measured by the conventional Warburg manometric technique and phosphorylation was determined according to the isotope-distribution method of LINDBERG AND ERNSTER[4]. Statistical evaluation of the data involved a paired comparison method[5].

In the aging studies mitochondrial suspensions were incubated in the absence of substrate and Mg^{++} for 0 to 25 min, a condition known to stimulate physical alteration of the mitochondrial structure[6], and subsequently incubated with succinate for 20 min in order to measure their phosphorylative capacity. The data are expressed as the "aging index^{P}" which is defined as the time of aging, in min, required to depress the phosphorylative capacity of the mitochondria to a value which is half way between the maximal and minimal phosphorylation.

The results of the P/O and aging studies are reported in Table I. Although P/O ratios obtained with mitochondrial preparations of epinephrine-treated rats do not differ from their controls, mitochondria from the former are considerably more resistant to the uncoupling effects of aging. This resistance is greater than would be inferred from the data since in many experiments uncoupling of these preparations had not occurred at the termination of the experiment. In experiments in which loss of phosphorylation had not occurred within the 25-min aging period, the "aging index^{P}" was taken as 25. This practice would result in mean values somewhat lower than actuality. For further information on this tendency, P/O and aging experiments were repeated with mitochondria from adrenal-demedullated rats. The results re-

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[*] Taken from a thesis by Dr. LIANIDES submitted to Tufts University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

TABLE I

MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION IN EPINEPHRINE-TREATED AND DEMEDULLATED RATS

Each incubation vessel contained: mitochondria, about 1/20th rat liver; orthophosphate, 50 μmoles; KCl, 150 μmoles; adenylic acid, 4.3 μmoles; glucose, 60 μmoles; sucrose, 125 μmoles; succinate, 30 μmoles; Mg^{++}, 7.5 μmoles; hexokinase, 330 K.M. units in P/O studies and 660 K.M. units in aging studies. Final volume, 2.0 ml. Gas phase, air. Temperature, 31°. Time of incubation, 15 or 20 min.

Type of study	*Rats injected with*		*P*	*Conditions of rats*		*P*
	Saline	*Epinephrine*		*Sham-operated*	*Adrenal demedullated*	
P/O	1.59	1.63	>0.56	1.73	1.69	0.43
Aging index^{P}	16.1	>22.6	0.03	16.8	10.9	0.04

corded in Table I reveal no difference in the P/O ratios of mitochondria from sham-operated and demedullated rats, results agreeing with those obtained with epinephrine-treated animals. On the other hand, the "aging index^{P}" of mitochondria from demedullated rats is lower than that of intact ones, indicating that oxidative phosphorylation is uncoupled more readily in preparations from animals lacking adrenal medullary secretions. These experiments strongly suggest that the adrenal medulla does not promote uncoupling of oxidative phosphorylation; conversely it appears to increase mitochondrial resistance to the uncoupling effects of aging. The physiological significance of this finding requires further investigation.

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[1] J. H. PARK, B. P. MERIWETHER AND C. R. PARK, *Federation Proc.*, 15 (1956) 141.
[2] G. F. MALEY AND H. A. LARDY, *J. Biol. Chem.*, 210 (1957) 903.
[3] W. C. SCHNEIDER AND G. H. HOGEBOOM, *J. Biol. Chem.*, 183 (1950) 123.
[4] O. LINDBERG AND L. ERNSTER, *Methods of Biochem. Anal.*, 3 (1956) 1.
[5] G. W. SNEDECOR, *Statistical Methods*, Iowa State College Press, Ames, Iowa, 4th Ed., 1946, p. 31.
[6] L. ERNSTER AND H. LÖW, *Exptl. Cell Research, Suppl.*, 3 (1955) 133.

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Glycogenolysis in liver slices of lactose-fed rats

Liver glycogen is known to increase after lactose feeding[1,2] and disappears in galactose-fed dogs more slowly than in glucose-fed animals[1]. It has also been noted that a hyperglycaemic state prevails in lactose-fed rats due to the presence of galactose in addition to glucose in blood[3]. It is possible that hyperglycaemia in lactose feeding is responsible for the slowing down of liver glycogen breakdown.

In the present investigation the rate of liver glycogenolysis of lactose-fed rats and of normal rats has been studied *in vitro* in a sugar-free medium.

Albino rats weighing between 35 and 40 g were divided in two groups. Group I was fed a diet containing 70 % sucrose, 15 % casein, 5 % cod-liver oil, 5 % cocoanut oil, 4 % salt mixture[4] and 1 % yeast. In group II, 50 % sucrose and 20 % lactose replaced the 70 % sucrose. Feeding was continued for 30 to 37 days, when the rats were sacrificed and the rate of liver glycogenolysis was measured according to the method of SUTHERLAND AND CORI[5].

Results given in Table I show that the rate of glycogenolysis in liver is slower in group II than in group I. Mean values of glucose liberation in 40 min by 100 mg liver tissue were 0.701 ± 0.036 mg and 0.406 ± 0.060 mg for groups I and II, respectively. Hence, it is concluded that in lactose-fed rats glycogenolysis in liver is slow even in a sugar-free medium indicating that slowing of liver glycogenolysis in lactose-fed rats is not due to the hyperglycaemic condition prevailing.

The authors are grateful to Principal K. C. MUKHERJEE for his interest and advice.

TABLE I

RATE OF GLYCOGENOLYSIS IN LIVER SLICES FROM RATS FED NO LACTOSE (GROUP I) OR FED 20 % LACTOSE FOR A PERIOD OF 30 TO 37 DAYS (GROUP I)

8 rats in each group. Values are means ± standard deviation.

Group	Glycogen (mg/100 mg liver tissue)	Glucose liberation (mg/100 mg liver tissue)			Glucose liberation (mg/40 min—a to c)	Glucose liberation (mg/mg of initial glycogen content/40 min — a to c)
		20 min (a)	40 min (b)	60 min (c)		
I	1.564 ± 0.06	0.66 ± 0.14	1.06 ± 0.06	1.39 ± 0.08	0.701 ± 0.036	0.448 ± 0.02
II	1.903 ± 0.08 F = 15	0.54 ± 0.10	0.77 ± 0.10	0.94 ± 0.11	0.406 ± 0.060 F = 12	0.213 ± 0.03 F = 35

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[1] H. J. DEUEL, JR., E. M. MACKNEY, P. W. JEWEL, M. GULLICK AND C. F. GREENEWALD, *J. Biol. Chem.*, 101 (1933) 301.
[2] M. N. COREYLL AND A. A. CHRISTMAN, *J. Biol. Chem.*, 150 (1943) 143.
[3] D. P. SADHU AND ANUBHA CHOWDHURY, *Indian J. Physiol. and Allied Sci.*, 10 (1956) 165.
[4] R. B. HUBBELL, D. B. MENDEL AND A. J. WAKEMEN, *J. Nutrition*, 14 (1937) 273.
[5] E. W. SUTHERLAND AND C. F. CORI, *J. Biol. Chem.*, 172 (1948) 737.

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Reactions of uridine diphosphate sugars catalyzed by enzymes of French-bean leaves

Many compounds containing uridine diphosphate linked to sugars have now been isolated from plants, and these sugar nucleotides have been shown to undergo a variety of conversions and to act as glycosyl donors in the presence of plant enzymes[1]. Whereas D-glucuronic acid occurs in plants chiefly as a constituent of complex polysaccharides such as hemicelluloses and gums, in a few instances simple conjugates with flavones, of similar structure to the phenolic β-D-glucopyranosiduronic acids excreted in mammalian urine, have been isolated[2]. It was thus of interest to study the reactions undergone by UDPGA in the presence of plant extracts, and to determine whether an enzyme analogous to the UDP-glucuronyl transferase in certain mammalian tissues[3] was present.

All the experiments were carried out with radioactive UDP-sugars labelled uniformly with ^{14}C in the terminal sugar moiety and of identical specific activity (37 μC/μmole)[4]. The techniques of incubation in sealed capillary tubes, electrophoresis of the reaction products on strips of acid-washed Whatman No. 1 filter paper, and location of radioactive spots by autoradiography were those of HASSID *et al.*[5]. Reaction products after elution were generally identified by coelectrophoresis, and in specific cases by cochromatography on paper, with authentic compounds.

Soluble enzyme preparations were prepared from French beans (*Phaseolus vulgaris*, variety unspecified), the young greenhouse plants being gathered just prior to flowering*. The washed leaves were homogenized in 0.01 *M* phosphate buffer, pH 7, containing 0.01 *M* mercaptoethanol. After being centrifuged at 20,000 × *g* for 30 min, the fraction of the supernatant which precipitated between 30–70 % ammonium sulphate saturation was redissolved in the minimum quantity of 0.1 *M* tris(hydroxymethyl)aminomethane buffer, pH 7.0, containing 0.1 *M* mercaptoethanol and dialysed against the same buffer for 4 h. All operations were carried out at 0°, and such preparations contained about 30 mg protein/ml[6].

[^{14}C]UDPGA ($1.6 \cdot 10^{-3}$ μmoles, 5000 counts/min) was incubated with the enzyme solution (20 μl) in a total volume of 40 μl containing 0.005 *M* $MgCl_2$ for 2 h at 37°, and the products separated by electrophoresis at pH 3.6 in 0.1 *M* ammonium formate buffer. There was complete disappearance of UDPGA, with formation of GA, GA-1-P, xylose and arabinose. Inclusion of UTP (0.5 μmole) in the incubation mixture decreased the rate of UDPGA decomposition, 25 % being retained; UDP or ATP had similar but less pronounced effects. Probably UDPGA is destroyed by nucleotide pyrophosphatases, and other nucleotides protect by competition. However, UTP may have an additional effect, for when [^{14}C]GA-1-P ($0.8 \cdot 10^{-3}$ μmole) was substituted for UDPGA in the system containing UTP together with addition of a conc. inorganic pyrophosphatase preparation, after 4 h incubation at 37° UDPGA was synthesised in 60 % yield. Hence it appears that a UDPGA pyrophosphorylase is present in the bean preparation, causing reformation of UDPGA in the experiments above; this enzyme has previously been reported in plants[7].

Abbreviations: UTP, uridine 5′-triphosphate; UDP, uridine 5′-diphosphate; UDPGA, uridine diphosphate glucuronic acid; UDPGalA, uridine diphosphate galacturonic acid; GA, glucuronic acid; GA-1-P, α-glucuronic acid 1-phosphate.

* Supplied by the Department of Botany, University of California (Davis), to whom the author is indebted.

In some experiments where UTP was included, further radioactive spots of mobility 1.0–1.2 with respect to picrate were noted after electrophoresis. These were eluted and tentatively identified as UDP-pentose and pentose 1-phosphate. Hydrolysis of these compounds with 1 *N* HCl at 100° for 30 min yielded a mixture of xylose and arabinose, but the parent UDP-pentoses could not be separated by paper chromatography.

Quercetin (10 μl satd. solution, pH 7.0, containing 0.1 μmole) was added to the mixture containing enzyme, [^{14}C]UDPGA, Mg^{++} and UTP and the period of incubation increased to 4 h. On electrophoresis, 42 % of the total radioactivity had zero mobility, and was partly adsorbed on to or incorporated into insoluble material. Elution of this with water removed only pentose (7 % of the original radioactivity); elution of the residue with 0.05 *M* ammonium carbonate, pH 9.0, at 0° for 6 h removed 8 % of the original radioactivity. This latter fraction was then found to be identical with authentic quercetin β-D-glucosiduronic acid[2] in electrophoretic mobility at pH 5.8 (mobility, 0.28) and pH 9.0 (mobility, 0.71) and by ascending chromatography in *n*-butanol–acetic acid–water (4:1:5) (R_F, 0.66). It was completely hydrolysed by a partially purified β-glucuronidase preparation from female-rat preputial gland[8] whereas in the presence of 0.001 *M* boiled D-glucosaccharic acid, a specific inhibitor of β-glucuronidase[9], 85 % of the radioactivity retained its original electrophoretic mobility. It thus seems probable that there had been transfer of glucuronic acid to quercetin to form a β-glucuronide identical with that previously isolated from the French bean[2]. The same compound was obtained when [^{14}C]GA-1-P together with UTP, but not with ATP or alone, was substituted for UDPGA. The nature of the residual non-mobile and insoluble radioactive material, in incubates containing quercetin, is unknown, but a water-insoluble product containing xylose and presumably lipid material has been reported in transfer reactions involving UDP-xylose[10].

The UDP-glucuronyl transferase system in mammalian tissues has wide acceptor specificity, but when phenol, *l*-menthol, benzoic acid, GA, GA-1-P, D-xylose or D-xylose 1-phosphate were substituted for quercetin in the above experiments, no evidence of the transfer of glucuronic acid could be obtained; YAMAHA AND CARDINI[11, 12] were unable to demonstrate transfer of GA from UDPGA to hydroquinone or phenyl β-glucoside, using a wheat-germ extract.

When [^{14}C]UDPGalA was incubated with the bean-enzyme preparation in the presence of Mg^{++}, galacturonic acid was the sole reaction product. When UTP was included, galacturonic acid 1-phosphate (90 % yield) was produced after 3 h incubation at 37°; the further addition of quercetin had no effect and gave no evidence of glucuronic acid conjugation with the flavonol. As pentoses were not formed in these experiments, it appeared that no UDPGalA 4-epimerase or UDPGalA decarboxylase was present. The occurrence of arabinose as a decomposition product of UDPGA thus probably arises from the formation of UDP-arabinose by action of a UDP-pentose 4-epimerase on UDP-xylose which results from UDPGA decarboxylation[13].

It is of interest that, despite the existence of the five free hydroxyl groups in the quercetin molecule, only the mono-β-D-glucuronide appears to be formed; in other compounds of this type the C-7 position of the flavone nucleus seems to be the site of conjugation[14].

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[1] W. Z. HASSID, E. F. NEUFELD AND D. S. FEINGOLD, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 905.
[2] C. A. MARSH, *Nature*, 176 (1955) 176.
[3] G. J. DUTTON AND I. D. E. STOREY, *Biochem. J.*, 57 (1954) 275.
[4] E. F. NEUFELD, D. S. FEINGOLD AND W. Z. HASSID, *J. Am. Chem. Soc.*, 80 (1958) 4430.
[5] D. S. FEINGOLD, E. F. NEUFELD AND W. Z. HASSID, *J. Biol. Chem.*, 233 (1958) 783.
[6] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
[7] D. S. FEINGOLD, E. F. NEUFELD AND W. Z. HASSID, *Arch. Biochem. Biophys.*, 78 (1958) 401.
[8] G. A. LEVVY, A. MCALLAN AND C. A. MARSH, *Biochem. J.*, 69 (1958) 22.
[9] G. A. LEVVY, *Biochem. J.*, 52 (1952) 464.
[10] D. S. FEINFOLD, E. F. NEUFELD AND W. Z. HASSID, *J. Biol. Chem.*, 234 (1959) 488.
[11] T. YAMAHA AND C. E. CARDINI, *Arch. Biochem. Biophys.*, 86 (1960) 127.
[12] T. YAMAHA AND C. E. CARDINI, *Arch. Biochem. Biophys.*, 86 (1960) 133.
[13] D. S. FEINGOLD, E. F. NEUFELD AND W. Z. HASSID, *J. Biol. Chem.*, 235 (1960) 910.
[14] K. SHIBATA AND S. HATTORI, *Acta Phytochim. (Japan)*, 5 (1930) 117.

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The binding of Fe^{+++} to native and chemically modified human serum albumin in the presence of sodium citrate

CAMON[1] studied the binding of Fe^{+++} to bovine serum albumin over the pH range 1.5 to 3.5. His ultrafiltration experiments showed that a maximum binding of 38 atoms/mole occurred at pH 3.5 in a medium with a free-Fe^{+++} concentration of $1.7 \cdot 10^{-3}$ *M*. Ferric hydroxide precipitation at a more alkaline pH effectively limited his study. WARNER AND WEBER[2] studied Fe^{+++} binding to conalbumin in the pH range 3 to 11 and overcame precipitation by complexing the iron with citrate. They used citrate in their dialysis systems but did not find it as part of the conalbumin–iron complex. For purposes of calculation, however, they assumed a citrate–iron complex of composition 1:1 as their source of iron. BOBTELSKY AND GOLDSCHMIDT[3] studied simple complex formation between Fe^{+++} and citrate ions. Their results suggested that in the pH range 3 to 5 the complex had the composition 2 Fe^{+++}: 3 citrate ions with a net negative charge of three.

We have studied Fe^{+++} binding at near physiological pH. In view of the evidence quoted we have attempted to measure both the iron and citrate of the protein complex. Our experimental method was equilibrium dialysis at room temperature. The pH range used was 3.0 to 7.3 and the Fe^{+++} concentration was $1.8 \cdot 10^{-4}$ *M*. The buffer systems used were: pH 3.0 to 3.8, 0.05 *M* potassium hydrogen phthalate–HCl; pH 3.8 to 5.8, 0.05 *M* sodium acetate–acetic acid; pH 5.7 to 7.3, 0.01 *M* sodium cacodylate.

Each buffer was adjusted to an ionic strength of 0.16 to 0.18 with NaCl. Sodium citrate was added to give either a 1:1 or 1:5 iron: citrate ratio as required. Fe^{+++} was added as $Fe(NO_3)_3$ and tracer ^{59}Fe. All iron and citrate estimations were by isotope dilution. Protein was assayed by micro-Kjeldahl. For calculations the molecular weight of human albumin was taken as 65,500. In addition to native albumin two chemically modified albumins were studied. Acetylated albumin with 70% of the amino groups blocked was prepared by the method of MARRACK[4]. 100%-esterified albumin was prepared by a modification of FRAENKEL-CONRAT AND OLCOTT's[5] method.

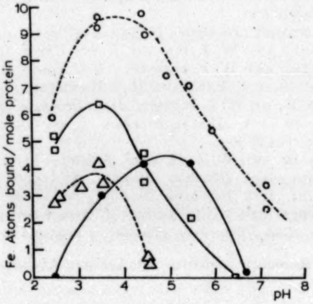

Fig. 1. ○, ●, variation of iron binding to esterified albumin; □, △, variation of native human serum albumin with pH. Solid lines represent experiments with a citrate–iron ratio 1:1. Dotted lines represent experiments using a ratio of 5:1.

The effect of pH on Fe^{+++} binding to native albumin is shown in Fig. 1. Maximum binding occurs at pH 3.9. By increasing the ratio citrate–iron from 1:1 to 5:1 the maximum iron binding is decreased. This suggests that either the albumin and citrate compete for the available iron, or that albumin combines more strongly with an iron–citrate complex of low citrate composition rather than with one with high citrate content.

We have attempted to identify the site of binding of the iron, or its citrate complex, by studying various chemically modified albumins. Acetylated albumin did not bind iron in the pH range 3.8 to 7.4. This modification has an isoelectric point which is more acid than native albumin and so our observations suggest that iron is bound to a positively charged grouping on the protein. 100%-esterified albumin showed greater binding than native albumin. This modification has an alkaline isoelectric point and would again be expected to combine predominantly with a grouping of net negative charge. Our evidence thus suggests that the iron is first complexed with citrate and that this complex, with a net negative charge, combines with the protein at some point of positive charge, such as an amino group. By decreasing the citrate–iron ratio from 5:1 to 1:1 the esterified albumin binding is considerably decreased (see Fig. 1). This suggests that the complex to be expected for binding has a citrate–iron ratio in excess of 1:1. A similar change in ratio has the opposite effect for native albumin.

We decided to measure the binding of [^{14}C]citric acid to albumin in the presence of inactive iron. At the same time an identical experiment was tried using inactive citrate and labelled iron. The [^{14}C]citric acid was measured by the β-scintillation technique using a solid phosphor as tried by STEINBERG[6]. For native albumin the ratio of protein-bound citrate to bound Fe^{+++} is 1.5 (mean of 6 experiments). This confirms our postulate that the simple complex of BOBTELSKY AND GOLDSCHMIDT, of composition $(2Fe:3Cit)^{---}$, combines with native human serum albumin. Similar experiments with esterified albumin gave an average of 1.1 citrate ions for each Fe^{+++} bound (average of 6 experiments). We conclude that the combination with esterified albumin is in some way different from that with native albumin.

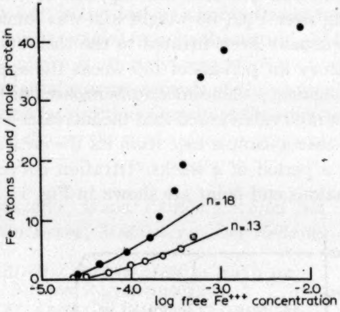

Fig. 2. ○, the variation of ferric ion binding to native albumin; ●, esterified albumin with free ferric ion concentration. The solid lines represent theoretical curves for the number of binding sites indicated by n.

Experiments to assess the number of binding sites on these two proteins confirm these conclusions. Fig. 2 shows the number of atoms of iron bound/mole protein at pH 3.9 with increasing Fe^{++} concentration. For native albumin it is seen that the theoretical graph for 13 binding sites fits the experimental points. For esterified albumin, however, the theoretical binding graph for 18 similar sites does not fit the experimental points at Fe^{+++} concentrations in excess of $10^{-3.5}$ *M*. The interpretation of this is that sites of a different type become available beyond a Fe^{+++} concentration in excess of 10^{-4} *M*. We conclude that for native human serum albumin there are sites available to complex approximately 13 Fe^{+++} in the form of citrate complexes of composition $(2Fe^{+++}3Cit^{---})^{---}$. For esterified albumin there are many more sites available for a citrate-iron complex which is of uncertain composition.

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[1] L. CAMON, *Rev. acad. cienc. exact. fis. quim. y nat., Zaragoza*, 11 (1956) 35.
[2] R. C. WARNER AND I. WEBER, *J. Am. Chem. Soc.*, 75 (1953) 5094.
[3] M. BOBTELSKY AND J. M. E. GOLDSCHMIDT, *Bull. Research Council Israel, Sect. A*, 7 (1958) 121.
[4] J. R. MARRACK, *Brit. J. Exptl. Pathol.*, 35 (1954) 389.
[5] H. FRAENKEL-CONRAT AND H. S. OLCOTT, *J. Biol. Chem.*, 161 (1945) 259.
[6] D. STEINBERG, *Nature*, 183 (1959) 1253.

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Anomalous titrations with cysteine

It has already been reported by SLUYTERMAN[1] and by STAIB AND TURBA[2] that cysteine titrated amperometrically against Ag^+ shows 1.3–1.6 times its theoretical -SH content. Similar findings have now been made in the case of potentiometric titrations of cysteine hydrochloride against *p*-chloromercuribenzoic acid for -SH determinations by the method of CALCUTT AND DOXEY[3].

The cysteine hydrochloride used in this work has been obtained in small lots from British Drug Houses Ltd., London. This was supplied as containing not more than 1 % water, but on drying over P_2O_5 no weight loss was found. Samples as received from the suppliers have usually been titrated to the theoretical -SH content. After being held in the laboratory for periods of 1–6 weeks the apparent -SH content by potentiometric titration against *p*-chloromercuribenzoate has been found to increase. Further titrations at later intervals showed that the increase—once it had appeared—was progressive. In one case a sample rose from its theoretical -SH content to 2.25 times this figure during a period of 4 weeks. Titration curves for samples giving a theoretical and an anomalous end point are shown in Fig. 1.

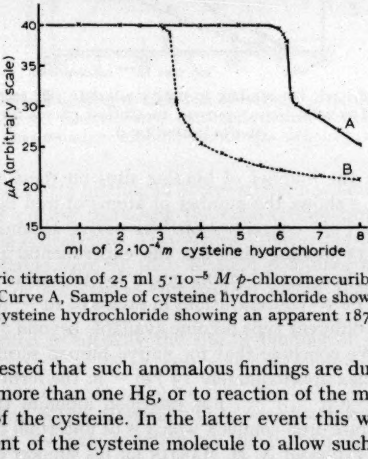

Fig. 1. The potentiometric titration of 25 ml $5 \cdot 10^{-5}$ *M* *p*-chloromercuribenzoate against $2 \cdot 10^{-4}$ *M* cysteine hydrochloride. Curve A, Sample of cysteine hydrochloride showing theoretical end point; curve B, Sample of cysteine hydrochloride showing an apparent 187% of theoretical -SH.

It has been suggested that such anomalous findings are due, either to reaction of the -SH group with more than one Hg, or to reaction of the mercury with the amino or carboxyl groups of the cysteine. In the latter event this would presuppose some internal rearrangement of the cysteine molecule to allow such reaction. It is widely recognised that internal rearrangements of molecules can be brought about by heat. Considerations of conditions in the laboratory showed that cysteine samples were being submitted to temperatures up to 35° during storage.

This possibility of effects due to heating was tested experimentally. A sample of cysteine which titrated to its correct -SH content was divided into two lots. One lot was maintained in the dark at a temperature below 20° whilst the other lot was incubated at 37° in the dark. At successive daily intervals samples were taken from the two lots and titrated against *p*-chloromercuribenzoate. Over a period of 4 days the lot left at 20° showed no deviations from its theoretical -SH content but the incubated lot titrated as 1.03; 1.07; 1.09 and 1.15 times the theoretical content over the same period. The possibility of similar effects due to heat in commercial samples is obvious from the fact that a common method of drying cysteine (and its hydrochloride) is by warming in a stream of nitrogen.

If internal rearrangement does occur in cysteine then solution and recrystallisation might be expected to restore the original state. A sample of cysteine hydrochloride which had changed to give 2.1 times its theoretical -SH content was dissolved in distilled water, concentrated *in vacuo* and precipitated with conc. HCl. The precipitate was filtered off and divided into two lots. One was dried in a stream of warm nitrogen and on titration gave an -SH content of 1.3 times the theoretical. The second lot was dried by successive washes of acetone and finally ether. This lot on titration gave a theoretical -SH content. Left in the dark at a normal room temperature (not over 22°) this particular sample has not varied from its theoretical -SH content during 8 weeks.

The evidence given above is strongly suggestive that anomalous cysteine titrations are the result of internal rearrangements of the molecule leading to availability of amino or carboxyl group for reaction. Certainly, the evidence indicates the necessity for the avoidance of heat during the final stages of preparation or storage of cysteine or its hydrochloride.

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[1] L. A. AE. SLUYTERMAN, *Biochim. Biophys. Acta*, 25 (1957) 402.
[2] W. STAIB AND E. TURBA, *Biochem. Z.*, 327 (1956) 475.
[3] G. CALCUTT AND D. DOXEY, *Exptl. Cell Research*, 17 (1959) 542.

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Reduction of protein disulfide bonds by sodium borohydride

Although a number of substances have been used for the reduction of disulfide bonds in proteins there still exists a need for more satisfactory means of reducing such disulfides quantitatively[1]. MOORE *et al.*[2] described the use of $NaBH_4$ to reduce quantitatively disulfides in a number of proteins in 8 M urea. This paper describes the use of $NaBH_4$ for the quantitative reduction of disulfide bonds in proteins in aqueous solution in the presence and absence of the denaturant, sodium dodecyl sulfate.

Reagents and proteins were of commercial origin except whale myoglobin which was isolated from muscle tissues by ammonium sulfate precipitation as described previously[3]. The extent of the reduction was determined by measuring sulfhydryl concentration by amperometric titration[3,4]. All titrations were conducted in a nitrogen atmosphere; reduced glutathione served as a standard. Titration values were corrected by a blank value (10 % or less of total) obtained by titrating a solution containing $NaBH_4$, buffer and other additives, but no disulfide-containing material. The titrations were readily reproducible and the standard titration value was always within 5 % of theory.

Reduction of the proteins and other materials was achieved by combining solutions of the reactants (0.10–2.00 μmoles protein + 1 ml of a 25 mg/ml freshly

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Abbreviation: SDS, sodium dodecyl sulfate.

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prepared solution of $NaBH_4$, total volume 5 ml) in a small beaker. When SDS was included in the mixture it was added from a 5 % stock solution to give a final concentration of 0.5 %. A minute amount of Dow Antifoam was added and the mixture incubated under a gentle stream of nitrogen in a water bath at the desired temperature. Under these conditions the reduced proteins remained soluble. At the end of the incubation period the material was washed into the titration vessel which was being flushed with nitrogen. Buffer and other components of the titration mixture were added, and the titration started after an equilibration period of about 5 min. The excess $NaBH_4$ was destroyed by the addition of 2 ml acetone[5].

Results are shown in Table I. Values are averages of at least duplicate determinations and have been rounded to the nearest 5 % titratable –SH. Control reductions done on oxidized glutathione, cystine, and lipoic acid gave theoretical values. Whale myoglobin, which has no sulfhydryl or disulfide, served as a negative control for the reduction and showed the expected zero titration. Since aqueous solutions of $NaBH_4$ are alkaline, the titrations of proteins treated with SDS at pH 9.0 were also included as controls.

TABLE I

$NaBH_4$ REDUCTION OF DISULFIDES IN PROTEINS

Protein and treatment[*]	*Percentage*[*] *of total –SH titratable after:*			
	5 min	*15 min*	*30 min*	*60 min*
Ribonuclease				
+ SDS, 40°			0	
+ SDS, pH 9.0, 40°			0	
+ BH_4^-, 40°			35	
+ BH_4^-, 45°			80	
+ BH_4^-, 50°			100	
+ BH_4^- + SDS, 40°	15	15	65	80
+ BH_4^- + SDS, 45°	45	75	95	100
Pepsin				
+ SDS, 40°			0	0
+ SDS, pH 9.0, 40°			0	
+ BH_4^-, 40°		35	95	95
+ BH_4^- + SDS, 40°	10	45	95	100
Ovalbumin				
+ SDS, 40°		15		20
+ SDS, pH 9.0, 40°				10
+ BH_4^-, 40°				40
+ BH_4^- + SDS, 40°	40	60	75	100
Bovine plasma albumin				
+ SDS, 50°			0	
+ SDS, pH 9.0, 50°			0	
+ BH_4^-, 50°	30	75	100	
+ BH_4^-, 40°			85	100
+ BH_4^- + SDS, 40°			see text	

[*] Treatment refers to conditions of incubation of proteins with SDS and/or BH_4^- at indicated temperature.

[**] Percentage total –SH based on total disulfides/mole of: ribonuclease, 4 per mol. wt. of 13,700; pepsin, 6 per 34,400; ovalbumin, 1 per 45,000 (plus 4–SH); bovine plasma albumin, 17 per 69,000 (plus 1–SH).

The results indicate that $NaBH_4$ can be used to quantitatively reduce disulfides in several proteins in aqueous solutions under relatively mild conditions. Generally, slightly milder conditions could be used for the reduction in the presence of SDS. The only difficulty with apparent reoxidation in the presence of the detergent was in the case of bovine plasma albumin where quantitative results could not be repeatedly obtained with systems containing SDS. However in this case complete reduction was obtained by treatment with $NaBH_4$ alone.

The use of $NaBH_4$ appears to have distinct advantages over other reagents commonly used for disulfide reduction in proteins: thioglycolic acid, mercaptoethanol and sulfite. Thioglycolic acid and mercaptoethanol, as any other thiols, have the disadvantage of making difficult the determination of the extent of the reduction. Additionally it has been reported recently by WHITE[6] that thiolation by polythioglycolides is a major side reaction during thioglycolate reduction of disulfide bonds in ribonuclease. The reaction of sulfite gives one sulfhydryl plus one S-sulfonate per disulfide, whereas $NaBH_4$ treatment yields two sulfhydryls. The extent of reduction by sulfite can be followed titrimetrically, but it is difficult to determine when there has been complete conversion of each half-cystine to S-sulfonate. Furthermore the reaction with sulfite also must be conducted in 8 *M* urea solutions to achieve completion[7,8].

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[1] R. CECIL AND J. R. MCPHEE, *Advances in Protein Chem.*, 14 (1959) 271.
[2] S. MOORE, R. D. COLE, H. G. GUNDLACH AND W. H. STEIN, *Intern. Congr. Biochem. 4th Meeting, Symposium No. 8, Vienna, 1958*, Pergamon Press, New York.
[3] A. M. DOLLAR, W. D. BROWN AND H. S. OLCOTT, *Biochem. Biophys. Research Communs.*, 1 (1959) 276.
[4] R. E. BENESCH, H. A. LARDY AND R. BENESCH, *J. Biol. Chem.*, 216 (1955) 663.
[5] H. C. BROWN, E. J. MEAD AND R. C. SUBBA RAO, *J. Am. Chem. Soc.*, 77 (1955) 6209.
[6] F. H. WHITE, JR., *J. Biol. Chem.*, 235 (1960) 383.
[7] J. L. BAILEY AND R. D. COLE, *J. Biol. Chem.*, 234 (1959) 1733.
[8] J. R. CARTER, *J. Biol. Chem.*, 234 (1959) 1705.

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Behavior of some selenium compounds in transmethylation

CANTONI AND MUDD[1] have shown that the selenium analogue of methionine is converted to "active selenomethionine" (Se-adenosylselenomethionine) at a rate which is similar to that at which "active methionine" is formed under identical conditions, and that when this Se-adenosylselenomethionine is incubated with guanidinoacetic acid and creatine methylpherase from pig liver, creatine is formed in excellent yield by transmethylation.

BREMER AND GREENBERG[2] have described a system in which choline biosynthesis is observed when [*Me*-^{14}C]S-adenosylmethionine is incubated with the microsome fraction of rat liver. In the present study, the ability of Se-adenosylselenomethionine to serve as methyl donor in this system was tested using [^{3}H]selenomethionine.

Selenomethionine, prepared in this laboratory according to the procedure described by PLIENINGER[3], was randomly tritiated by WILZBACK method[4] (New England Nuclear Corp., Boston), giving the specific activity of 2.12 mC/mg. Determination of the portion of radioactivity, associated with the methyl group of [^{3}H]-selenomethionine, was carried out as follows: S-methyl isothiourea picrate was prepared from 300 mg of non-radioactive carrier selenomethionine added to $1.375 \cdot 10^6$ counts/min of [^{3}H]selenomethionine, according to the procedure described by LEVINE AND TARVER[5]. The picrate (m.p. 221° after recrystallization from ethanol) was dissolved in conc. NH_4OH, and the methyl mercaptan, liberated by gentle heating, was led into a trap containing 4 % aq. $Hg(CN)_2$. The precipitate, $(CH_3S)_2Hg$, was recrystallized from chloroform (m.p. 175–176°). 2.2 mg of the mercury compound was counted in liquid scintillation counter to give 4,580 counts/min. Calculation showed that 28.7 % of the total activity in randomly labeled [^{3}H]selenomethionine was associated with the methyl group. [^{3}H]selenomethionine was converted to Se-adenosylselenomethionine according to the procedure used by CANTONI AND DURELL[6].

Table I shows an experiment in which S-adenosylmethionine and Se-adenosylselenomethionine were compared as substrate in choline biosynthesis. The selenium analogue was found to be as efficient a methyl donor as S-adenosylmethionine in this system.

TABLE I

SE-ADENOSYLSELENOMETHIONINE AS METHYL DONOR IN CHOLINE BIOSYNTHESIS

Radioactive substrate (1.5 μmoles, $6.14 \cdot 10^5$ counts/min** in S-adenosylmethionine and $3.10 \cdot 10^4$ counts/min in the methyl group of Se-adenosylselenomethionine) was incubated with rat-liver microsomes*** (24 mg protein) in glycine–KOH buffer (200 μmoles, pH 9.5) for 1 h at 37° in a total volume of 2 ml. The reaction was stopped by addition of 0.2 ml conc. HCl followed by 1.8 ml H_2O and 2 ml *n*-butanol.

Substrate	*Radioactivity in butanol extract**		*Net radioactivity representing phospholipid bases*
	Intact microsomes	*Boiled microsomes*	
[*Me*-^{14}C]S-adenosylmethionine	19.4	3.6	15.8
[^{3}H]Se-adenosylselenomethionine	59.6	37.4	22.2

* Expressed as percentage of added radioactivity found in products.

** The radioactivity determination in this work was done by Packard Tri-Carb liquid scintillation counter.

*** Preparation of microsomes and determination of protein were performed as reported previously[7].

As the incubation of Se-adenosylselenomethionine with boiled microsomes gave a relatively high activity with the *n*-butanol-extraction procedure used by BREMER AND GREENBERG, the following experiment was carried out to verify the formation of [^{3}H]choline. 1 ml of the butanol extract from the incubation mixture containing intact microsomes, which had 3,350 counts/min as phospholipid bases in the extract, was evaporated to dryness and subsequently refluxed for 4–5 h in an oil bath with 3–4 ml 2.5 *N* HCl and 25 mg carrier choline. After removing the liberated fatty acids by ether extraction, the choline was precipitated as the Reineckate and recrystallized three times from acetone with *n*-propanol. The choline was liberated from 10-mg samples of the Reineckate precipitate after each recrystallization by shaking the choline Reineckate with a mixture of 1 *N* HCl and methyl ethyl ketone. By this procedure the choline is extracted quantitatively into the aqueous phase while the liberated Reinecke salt is retained quantitatively in the methyl ethyl ketone phase. After evaporating the aqueous phase in the counting vials, the radioactivity of the choline residue was determined in the liquid scintillation counter. Specific activities of the Reineckate after one, two and three recrystallizations were 270, 245 and 253 counts/min/10 mg respectively. Considering that the molecular weight of choline Reineckate is 3.4 times that of choline chloride, at least 2,125 counts/min of the 3,350 counts in the original butanol extract must have been in the form of choline. This calculation does not take into account the unknown amount of choline present in the radioactive phospholipids extracted from the incubation mixture (presumably several mg) and it does not include the intermediate, mono- and di-methylaminoethanol, which are not coprecipitated with choline when the choline Reineckate is recrystallized.

In Table II is shown an experiment where S-adenosylmethionine and Se-adenosylselenomethionine were compared as methyl donors in the methylation of a sulfhydryl compound, 2-methoxyethanthiol, in a system identical to that used by Bremer and Greenberg[7]. Again, the selenium compound was found to be an effective methyl donor as estimated from the formation of radioactive toluene-soluble product, presumably O,S-dimethylmercaptoethanol.

TABLE II

Se-ADENOSYLSELENOMETHIONINE AS METHYL DONOR IN ENZYMIC METHYLATION OF A SULFHYDRYL GROUP

Radioactive substrate (0.75 μmole, $1.35 \cdot 10^5$ counts/min in S-adenosylmethionine and $1.46 \cdot 10^4$ counts/min in the methyl group of Se-adenosylselenomethionine) was incubated with rat-liver microsomes (10 mg protein) and 2-methoxyethanthiol (1 μl) in tris(hydroxymethyl)aminomethane buffer (50 μmoles, pH 8.0) at 37° in a total volume of 1 ml. Reaction was stopped by addition of 0.1 ml conc. HCl and the incubation mixture was extracted with toluene.

Substrate	*Volatile radioactivity in toluene extract**		
	20 min	*40 min*	*90 min*
$[Me\text{-}^{14}\text{C}]$S-adenosylmethionine	27.2	44.1	67.0
$[^{3}\text{H}]$Se-adenosylselenomethionine	13.4	24.1	40.7

* Expressed as percentage of added radioactivity found in products.

Challenger[8] and his group have observed that certain molds grown in culture containing inorganic selenate or selenite produce dimethylselenide, and S-adenosylmethionine has been inferred as principal methyl donor. In experiments *in vitro* with various animal tissues, conversion of inorganic selenate and selenide to "volatile selenium" has been observed[9]. Bremer and Greenberg[7] have shown that various sulfhydryl compounds can accept methyl group enzymically from $[Me\text{-}^{14}\text{C}]$S-adenosylmethionine. These findings led us to test H_2Se and methyl selenol as methyl acceptors in the microsomal system. As shown in Table III, these selenium compounds were found to be good methyl acceptors in enzymic methylation. The finding that some methylation takes place with boiled microsomes is in accordance with the observation

TABLE III

SELENIUM COMPOUND AS METHYL ACCEPTOR IN ENZYMIC METHYLATION

[*Me*-^{14}C]S-adenosylmethionine (0.7 µmole, 405,600 counts/min) was incubated with substrate (10 µmoles) and rat-liver microsomes (39 mg protein) in tris(hydroxymethyl)aminomethane buffer (100 µmoles, pH 8.0) for 2 h at 37° in a final volume of 1.9 ml. The reaction was stopped by addition of 0.2 ml conc. HCl. The methylation products were separated according to CHALLENGER[10]. 100–200 mg each of non-radioactive carrier, corresponding to the methylation products of the particular substrate, were added to the reaction mixture and the volatile gas was passed successively in a stream of air through (a) 4% aq. $Hg(CN)_2$ and (b) DIGINELLI's solution (10 g $HgCl_2$, 80 ml water, 20 ml conc. HCl) for 2 h with occasional swirling. 300–400 mg of the mercury salt of monomethyl compound was precipitated in (a) and 500–600 mg of the $HgCl_2$ salt of dimethyl compound was precipitated in (b). After recrystallization from benzene, the purity of the compounds was tested by melting-point determination***. In the experiment with H_2Se, the separation of the mono- and dimethyl compound was not attempted owing to the insolubility and heat lability of the methyl selenol mercury salt.

*Substrate**	*Methylation product*	*Radioactivity in methylation product***	
		Intact microsomes	*Boiled microsomes*
H_2S	CH_3SH	14.6	2.2
	CH_3SCH_3	1.8	0.4
H_2Se	$CH_3SeH + CH_3SeCH_3$	10.8	3.5
CH_3SeH	CH_3SeCH_3	11.7	3.0

* Aqueous solutions of Na_2S, Na_2Se and $NaSeCH_3$ were added as substrates.

** Expressed as percentage of added radioactivity found in products.

*** $(CH_3S)_2Hg$ m.p. 174–175°, $2(CH_3)_2S \cdot 3HgCl_2$ m.p. 157–158°, $(CH_3)_2Se \cdot HgCl_2$ m.p. 153–154° (decomp.). (These melting points are uncorrected.) These values correspond to those reported in ref. 10.

of STEKOL (private communication) that a nonenzymic transmethylation from S-adenosylmethionine to sulfhydryl compounds occurs.

The present work demonstrates that selenium analogues can replace some of the biologically important sulfur compounds in the enzymic methylation system found in rat liver.

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[1] S. H. MUDD AND G. L. CANTONI, *Nature*, 180 (1957) 1052.
[2] J. BREMER AND D. M. GREENBERG, *Biochim. Biophys. Acta*, 37 (1960) 173.
[3] H. PLIENINGER, *Ber.*, 83 (1950) [illegible].
[4] K. E. WILZBACH, *J. Am. Chem. Soc.*, 79 (1957) 1013.
[5] M. LEVINE AND H. TARVER, *J. Biol. Chem.*, 192 (1951) 835.
[6] G. L. CANTONI AND J. DURELL, *J. Biol. Chem.*, [illegible] (1957) 1033.
[7] J. BREMER AND D. M. GREENBERG, *Biochim. Biophys. Acta*, 37 (1960) 173.
[8] F. CHALLENGER, *Aspects of the Organic Chemistry of Sulphur*, Butterworth Scientific Publications, London, 1959, Chapter 5.
[9] I. ROSENFELD AND O. A. BEACH, *J. Biol. Chem.*, 172 (1948) 333.
[10] F. CHALLENGER, *Aspects of the Organic Chemistry of Sulphur*, Butterworth Scientific Publications, London, 1959, p. 18.

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A chitinase system from *Carcinus maenas*

The presence of chitinase activity in crustacean hypodermis is suggested by the partial resorption of chitin during moulting and by the observations of RENAUD[1] and KUHN AND TIEDMAN[2]. Using the crab *Maia squinado*, RENAUD[1] showed that extracts of the hypodermis released aminosugars from fragments of the integument, while KUHN AND TIEDMAN[2] described the hydrolysis of N-acetyl-β-glucosaminides by a lobster hypodermis preparation. Evidence of chitolytic enzymes in the hypodermis of the shore crab *Carcinus maenas* has now been obtained; the tissue is readily available and presents a convenient source of chitinase and N-acetyl-β-glucosaminidase.

Acetone-dried powders were prepared from the hypodermal tissue underlying the carapace of *C. maenas*, about 1 g of powder being obtained from 10 crabs. The powder could be stored at —14°, and retained both chitinase and N-acetyl-β-glucosaminidase activities for over a year. Enzymic extracts were prepared by mixing acetone powder with water (1:10, v/v) for 30 min with occasional stirring. The suspension was centrifuged (2,000 rev./min for 5 min) and the green-brown solution, after dialysis against distilled water (cellophane), was stored at —14° or 0° under toluene. Such preparations retained activity for over 6 months.

For use as a substrate in assays of enzymic activity, chitin was prepared in the following way: 368 mg of finely powdered chitin[3] were dissolved in 6 ml conc. HCl, and the viscous yellow solution diluted with 200 ml water. The precipitated chitin was collected by centrifugation, washed with 50 ml water, redissolved in HCl and precipitated as before. After two more washings, the chitin was suspended in water and thoroughly dispersed in a Potter-type homogenizer; it was then dialysed against running tap water (cellophane) for 40 h, and thrice against distilled water. The concentration of chitin in the suspension was determined on a dry-weight basis by drying (100° for 3 h), and weighing the solid obtained by centrifugation of a known volume of the suspension. The freshly shaken suspension contained 6.48 mg dry weight of chitin/ml and had an apparent extinction of 3.41 at 420 mμ (see ref. 4). The extinctions were measured in a Unicam SP 600 spectrophotometer using 1-cm cells. Using appropriate dilutions of this suspension, a linear relationship was established between the apparent extinction and chitin concentration over the range 0–1.3 mg/ml. The chitin content of incubation mixtures could thus be calculated.

The hydrolysis of phenyl and methyl N-acetyl-β-glucosaminides by the dialysed extracts in the presence of acetate or citrate buffer (pH 4.3) at 30° was followed. After incubation, proteins were removed by heating at 100° for 1 min and centrifuging; N-acetylglucosamine in the supernatants was estimated by the method of REISSIG, STROMINGER AND LELOIR[5]. It was found (Fig. 1) that the phenyl derivative was hydrolysed approximately 1.5 times faster than the methyl glycoside. The effect of pH on initial reaction velocities was investigated, and in a number of determinations (*e.g.*, see Fig. 2) optima between 4.3 and 4.8 were observed.

Chitinase activity was measured by incubating enzyme preparations with colloidal chitin and observing both the decrease in turbidity and the appearance of N-acetylglucosamine. The incubation mixtures consisted of 0.1 *M* phosphate buffer (pH 6.9), 0.1 ml; colloidal chitin suspension, 0.2 ml; enzyme preparation, 0.1 ml; toluene, 0.5 ml. At intervals, two tubes were withdrawn, their contents diluted to

3.0 ml, and after mixing, the apparent absorbancies at 420 mµ were measured. After heating at 100° for 1 min, proteins and unchanged chitin were removed by centrifugation, and the N-acetylglucosamine in the supernatants was determined. The results of one experiment are shown in Fig. 3. The hydrolysis products were examined by

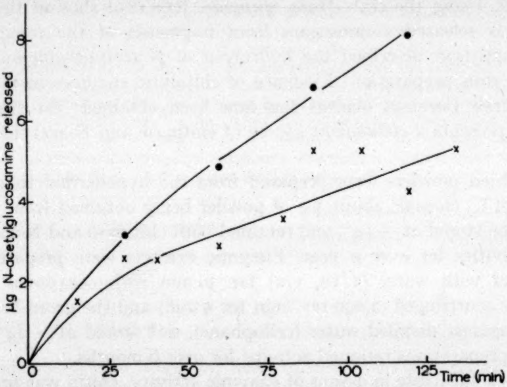

Fig. 1. Hydrolysis of glucosaminides. The incubation mixtures contained 0.1 ml dialysed extract, 0.2 ml of 0.067 *M* acetate buffer (pH 4.3), and 0.094 µmole of substrate, the final volume being 0.4 ml. X, methyl N-acetyl-β-glucosaminide; ●, phenyl N-acetyl-β-glucosaminide.

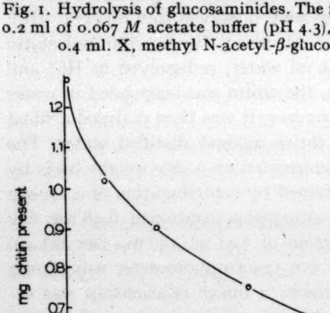

Fig. 2. Hydrolysis of colloidal chitin. Upper curve gives the fall in chitin content of the incubation mixtures, and the lower curve the increase in N-acetylglucosamine content over the same periods of time.

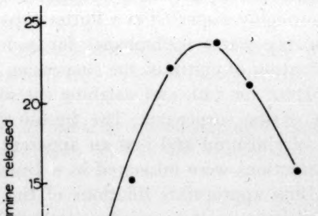

Fig. 3. The effect of pH on N-acetyl-β-glucosaminidase activity. The mixtures contained 0.1 ml of dialysed extract, 0.1 ml of 0.1 *M* citrate buffer and 0.1 µmole phenyl N-acetyl-β-glucosaminide, final volume being 0.4 ml. The incubation time was 30 min.

paper chromatography (phenol saturated with water) when N-acetylglucosamine appeared as the only substance giving a positive reaction with the Elson and Morgan spray reagent[6].

The results shown in Fig. 3 indicate that the release of N-acetylglucosamine from the chitin suspension stopped while the decrease in turbidity was still taking place. It therefore appears that possibly here, as in *Streptomyces griseus*[4], chitin breakdown may involve several enzymes, one of which causes depolymerization of the chitin chains, resulting in a lowering of the turbidities of chitin suspensions, another enzyme, which appears to be slowly inactivated at 37°, being responsible for the release of N-acetylglucosamine, either from the polysaccharides originally present, or from oligosaccharides produced enzymically. De-acetylation of the substrates was not observed.

Solutions of the *Carcinus* chitinase system have been used to demonstrate the release of N-acetylglucosamine from chitin samples obtained from several crustacean species by both the extraction method of BRACH[3], and the milder procedures of FOSTER AND HACKMAN[7].

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[1] L. RENAUD, *Ann. inst. océanogr. (Monaco)*, 24 (1949) 259.
[2] R. KUHN AND H. TIEDMAN, *Ber.*, 87 (1954) 1141.
[3] H. BRACH, *Biochem. Z.*, 38 (1912) 468.
[4] L. R. BERGER AND D. M. REYNOLDS, *Biochim. Biophys. Acta*, 29 (1958) 522.
[5] J. L. REISSIG, J. L. STROMINGER AND L. F. LELOIR, *J. Biol. Chem.*, 217 (1955) 959.
[6] S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238.
[7] A. B. FOSTER AND R. H. HACKMAN, *Nature*, 180 (1957) 40.

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Pathways of triglyceride formation in adipose tissue

The assimilation of fatty acids by adipose tissue takes place predominantly by transformation into triglycerides, and the rate of uptake of the acids from the medium was found to be correlated with the esterifying capacity of the tissue[1]. The following reactions may contribute to the incorporation of fatty acids into triglycerides: (a) The formation of an acyl-CoA compound, which then interacts with α-glycerophosphate and finally is converted into triglycerides by a series of reaction steps, demonstrated in liver tissue[2,3]. The presence of the enzymes for this reaction sequence in adipose tissue has been demonstrated[4]. (b) The acyl-CoA compound formed may interact with diglycerides present in the tissue to form directly a triglyceride. (c) A lipase-activated exchange reaction between triglycerides and free fatty acids may introduce some of the radioactive fatty acid into the triglyceride[5].

It was of interest to determine which of these three possibilities was the dominant

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Abbreviation: CoA, coenzyme A.

one, since this knowledge may be relevant for an understanding of the assimilation of fatty acids by adipose tissue, and of the nature of the acceptor for the fatty acids.

When radioactive palmitate is incorporated into the triglycerides, it is possible to differentiate between the first of these three alternatives and the other two, since with the last (b and c), only one fatty acid in the triglyceride, that in an α-position, will be labeled, while with the first set of reactions (a), all three hydroxy groups of the glycerol will be occupied by the labeled fatty acid.

In order to determine the distribution of labeled fatty acids in the triglycerides, the latter were subjected to degradation by pancreatic lipase. This lipase is known[6,7] to split triglycerides mainly in the α-position, forming 1,2-diglycerides and 2-monoglycerides. In the case of α-labeled triglycerides this degradation would yield diglycerides with decreased specific activity and monoglycerides, practically devoid of activity. With triglycerides labeled equally in all three positions, on the other hand, no decrease in specific activity will take place.

The method was first tested, using triglycerides labeled by exchange with [1-^{14}C]palmitic acid in the presence of pancreatic lipase, which labels predominantly the α-position (see ref. 5).

The triglyceride was isolated from the reaction mixture by extraction with alcohol–ether (3:1, v/v). The extract, after evaporation, was taken up in acetone and passed through a MgO column to remove free fatty acids. The glycerides in the acetone eluate were then fractionated on a silicic acid column[8] and the triglyceride fraction obtained. Lipolytic degradation was now carried out on the triglyceride by a method similar to that of MATTSON[6]. The labeled triglycerides were dissolved in a small amount of ether and added to a hot solution of 5% gum accacia in 1 *N* NH_4OH–NH_4Cl buffer, pH 8.0, and dispersed by homogenization in a small blendor.

TABLE I

DEGRADATION OF NEWLY SYNTHESIZED RADIOACTIVE TRIGLYCERIDES DERIVED FROM ADIPOSE TISSUE

Expt.	*Specific activity (counts/min/µequiv. ester)*		
	Before lipolysis	*After lipolysis and subsequent MgO chromatography*	
		20 min lipolysis approx. 40%	*30 min lipolysis approx. 60%*
1	322	333	—
2	1455	1405	—
3	460	406	347
4	1625	1635	1460

Degradation of pancreatin-catalyzed exchange-labeled triglycerides

Expt.	*Specific activity (counts/min/µequiv. ester)*		
	Before lipolysis	*After lipolysis and subsequent MgO chromatography*	
		20 min lipolysis approx. 40%	*30 min. lipolysis approx. 60%*
1	100	27.5	—
2	250	42	—

2 ml of the resulting emulsion were then incubated with 1.8 ml of the ammonium buffer, 0.2 ml 60 % $CaCl_2$ and 100 mg pancreatin (Nutritional Biochemicals Corp., Cleveland) dispersed in 2 ml water at 37° in a water bath with continuous shaking. Incubation was continued until the lipolysis was about 40–60 % complete (followed by determination of the ester bonds, according to STERN AND SHAPIRO[9]), to obtain a fair proportion of monoglycerides. The glycerides were then extracted and isolated as described above, except that no additional separation of glycerides on silicic acid was undertaken. For the actual test, triglycerides were synthesized by mesenteria of rats previously starved for several days and refed on a high-carbohydrate diet, two days prior to the experiment. The tissues were incubated with [1-^{14}C]palmitic acid in a medium of 5 % albumin in Ca^{++}-free Krebs–Ringer phosphate solution. After 2-h incubation the triglycerides were isolated as described before and subjected to similar lipolytic degradation.

The results summarized in Table I show that while the glycerides obtained from triglycerides labeled by lipase-catalyzed exchange lost the major part of their activity upon subsequent lipolysis, the newly synthesized adipose tissue triglyceride underwent practically no reduction in specific activity by this procedure.

These results indicate that the triglyceride synthesis in adipose tissue follows mainly pathway (a), *i.e.* total esterification of α-glycerophosphate with three molecules of fatty acid.

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[1] B. SHAPIRO, I. CHOWERS AND G. ROSE, *Biochim. Biophys. Acta*, 23 (1957) 115.
[2] S. B. WEISS AND K. P. KENNEDY, *J. Am. Chem. Soc.*, 78 (1956) 8550.
[3] Y. STEIN, A. TIETZ AND B. SHAPIRO, *Biochim. Biophys. Acta*, 26 (1957) 286.
[4] G. ROSE AND B. SHAPIRO, *Bull. Research Council Israel. Sect. A*, 9A (1960) 15.
[5] B. BORGSTRÖM, *Biochim. Biophys. Acta*, 13 (1954) 491.
[6] F. H. MATTSON AND L. W. BECK, *J. Biol. Chem.*, 219 (1956) 735.
[7] P. SAVARY AND P. DESNUELLE, *Biochim. Biophys. Acta*, 21 (1956) 349.
[8] B. BORGSTRÖM, *Acta Physiol. Scand.*, 25 (1952) 11.
[9] I. STERN AND B. SHAPIRO, *J. Clin. Pathol.*, 6 (1953) 158.

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Enzymic synthesis of glutaryl-coenzyme A

The activation of succinate to succinyl-CoA[1,2] and of malonate to malonyl-CoA[3-5] by an ATP- and CoA-dependent reaction is well documented. We have found that glutaryl-CoA is synthesized in animal tissues from glutarate in analogous manner according to reaction (1).

$$\text{Glutarate} + \text{ATP} + \text{CoA} \rightleftharpoons \text{glutaryl-CoA} + \text{ADP} + P_i \qquad (1)$$

Abbreviations: CoA, coenzyme A; ATP, adenosine triphosphate; GTP, guanosine triphosphate; ITP, inosine triphosphate; Tris, tris(hydroxymethyl)aminomethane; GSH, glutathione.

The system of KAUFMAN[1] (*cf.* Table I) was used to assay activation of dicarboxylic acids. It is seen that not only was glutarate converted to glutaryl-CoA in dog heart and muscle and rat and pigeon liver, but in all tissues the rate of glutarate activation was significantly greater than that of succinate and malonate.

TABLE I

ENZYMIC ACTIVATION OF DICARBOXYLIC ACIDS

The assay system[1] contained (in μmoles): Tris buffer, pH 7.5 (100), glutarate, malonate or succinate (100), crystalline ATP (10), CoA (0.2), GSH (20), $MgCl_2$ (8), neutralized $NH_2OH \cdot HCl$ (1000) and enzyme. Volume, 2.0 ml. Incubation, 30 min at 37°. Values are mμmole hydroxamic acid formed (measured as in ref. 1) per mg protein/30 min. Dog-heart and skeletal-muscle and pigeon-liver preparations were dialyzed 0.35–0.65 satd. $(NH_4)_2SO_4$ fractions of crude extract. Rat liver was the 54,000 × *g* supernatant of a 0.25-*M* sucrose homogenate.

Substrate	*Dog heart*	*Dog muscle*	*Pigeon liver*	*Rat liver*
Malonate	10.3	2.6	4.7 (0)*	11.5
Succinate	7.4	8.2	20.5 (3.4)	62.9
Glutarate	28.4	16.0	31.7 (45.6)	86.7

* After fractionation with ethanol, 40–70%, at —10°.

The glutarate-activating enzyme was shown to be a discrete enzyme, as expected from the reported inactivity of glutarate with purified succinate-activating enzymes[1,6] (P enzyme), by purification procedures. By ethanol fractionation, it was resolved completely from the malonate-activating enzyme and largely from the succinate-activating enzyme in pigeon liver (Table I). The glutarate enzyme was purified 5-fold from dog-skeletal muscle, with complete resolution of malonate and succinate activity, but became unstable, being inactivated after several days at —18°.

TABLE II

NUCLEOTIDE SPECIFICITY OF GLUTARATE-ACTIVATING ENZYME

Standard assay conditions. Pigeon-liver calcium phosphate gel eluate fraction (11.4 mg protein) specific activity 35 (glutarate), 8.8 (succinate). Values are rates relative to GTP. Values in parentheses refer to dog-muscle fraction.

Nucleotide	*Succinate*	*Glutarate*
GTP	100	100 (100)
ITP	74	85
ATP	15	92 (100)

The glutarate-activating enzyme, unlike the succinate-activating enzyme, is about equally reactive with ATP, GTP and ITP (Table II). The relative reaction rates for the succinate enzyme with GTP, ITP and ATP in pigeon liver and dog muscle are comparable to those (100:67:27) observed in guinea-pig liver[7] and to those measured in pig kidney and heart[2] (GDP:IDP = 100:44).

The glutarohydroxamic formed in the assay system was extracted under mild conditions using acetone[3] and placed on Whatman No. 1 paper. The chromatogram was developed in isoamyl alcohol–formic acid solvent[8] and after spraying with $FeCl_3$ one spot (R_F, 0.32) corresponding to authentic glutaromonohydroxamic acid was found. With water-saturated butanol[3] as solvent, a single Fe-positive spot (R_F, 0.48) was again found (authentic glutaromonohydroxamic acid, 0.49).

Further evidence for reaction (1) was obtained by measuring acylation of substrate amounts of CoA in presence of ATP and glutarate (Table III). It appears that after several minutes a steady-state concentration of glutaryl-CoA was reached, probably reflecting opposing activation and deacylation reactions. The disappearance of CoA in presence of ATP and enzyme is not explained by oxidation.

TABLE III

ENZYMIC ACYLATION OF CoA BY GLUTARATE

Test system as in Table I (GSH and NH_2OH omitted) with 1.0 μmole CoA and dog-muscle fraction (5.4 mg protein). Sulfhydryl measured as in ref. 1.

System	*— Δ -SH*		
	5 min	*10 min*	*20 min*
Complete	0.33	0.48	0.58
No glutarate	0.20	0.36	0.44
— Δ SH, corrected	0.13	0.12	0.14

The substrate specificity of the glutarate-activating enzyme was tested. Glutaconate reacted at 40 % of the rate of glutarate. The α-methyl, α,α-dimethyl, β-methyl, and β,β-dimethylglutarate derivatives were inert with the pigeon-liver enzyme and slightly reactive (30 %) with the dog-muscle enzyme. Adipate, muconate, β-hydroxyglutarate and β-hydroxy-β-methylglutarate were inert. (The latter gave positive hydroxylamine- and CoA-acylation tests in muscle and liver, probably due to some contaminant since the acylation product was not cleaved by purified β-hydroxy-β-methylglutarate-cleavage enzyme[9]. Neither was this product acetyl-CoA.)

These experiments show that the activation of malonate, succinate and glutarate is catalyzed by three different enzymes. The glutarate-activating enzyme presumably plays a role in initiating the oxidation of glutarate in tissues[10].

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[1] S. KAUFMAN, C. GILVARG, O. CORI AND S. OCHOA, *J. Biol. Chem.*, 203 (1953) 869.
[2] D. R. SANADI, D. M. GIBSON, P. AYENGAR AND M. JACOB, *J. Biol. Chem.*, 218 (1956) 505.
[3] O. HAYAISHI, *J. Biol. Chem.*, 215 (1955) 125.
[4] H. I. NAKADA, J. B. WOLFE AND A. N. WICK, *J. Biol. Chem.*, 226 (1957) 145.
[5] R. O. BRADY, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 993.
[6] S. KAUFMAN AND S. G. ALIVISATOS, *J. Biol. Chem.*, 216 (1955) 141.
[7] J. ADLER, S. F. WANG AND H. A. LARDY, *J. Biol. Chem.*, 229 (1957) 865.
[8] M. FLAVIN AND S. OCHOA, *J. Biol. Chem.*, 229 (1957) 965.
[9] B. K. BACHHAWAT, W. G. ROBINSON AND M. J. COON, *J. Biol. Chem.*, 216 (1955) 727.
[10] E. R. TUSTANOFF AND J. R. STERN, *Biochem. Biophys. Res. Commun.*, 3 (1960) 81.

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Preliminary Notes

Distribution of guanylic acid in RNA of yeast and tobacco mosaic virus

Ribonuclease T_1 which specifically splits the internucleotide bonds between 3′-guanylic acid and 5′-hydroxyl groups of adjacent nucleotides was purified from takadiastase[1], and it was suggested as an useful tool for obtaining information on the nucleotide arrangement in RNA[2].

RNA was prepared from tobacco mosaic virus as a single molecular species by the phenyl method of GIERER AND SCHRAMM[3], followed by precipitation with 1 *M* NaCl[4]. For comparison, highly polymerized yeast RNA (preparation no. 9) was prepared by the use of sodium dodecyl sulfate according to CRESTFIELD *et al.*[5], although it is a heterogeneous polynucleotide mixture[6]. Commercial yeast RNA (Schwarz Laboratories) was purified by deproteinization and compared with high-molecular-weight RNA in regard to its behavior with RNase T_1.

RNA was mixed with RNase T_1 in Tris buffer (0.05 *M*, pH 7.5), and incubated at 37° for 6 h. The released mononucleotides consist of guanosine 3′-phosphate and guanosine 2′,3′-cyclic phosphate. The cyclic nucleotide was converted to guanosine 2′-phosphate and guanosine 3′-phosphate by the incubation of the enzyme digest in 0.1 *N* HCl for 20 h at 2°. The resulting guanosine 2′-phosphate and guanosine 3′-phosphate were separated from other nucleotide components by paper chromatography with the Whatman No. 1 and the solvent[7]: satd. $(NH_4)_2SO_4$–water–isopropanol (79:19:2, v/v/v). Guanylic acid was eluted in 0.1 *N* HCl and determined spectrophotometrically. The amount of the guanine mononucleotide was expressed as per cent of the total guanylic acid in RNA and of the total nucleotides in RNA, based on phosphorus analysis in the digest. Nucleotide composition was determined as described previously[8].

TABLE I

NUCLEOTIDE COMPOSITION AND GUANINE MONONUCLEOTIDE IN RNase T_1 DIGEST

RNA preparation	*Nucleotide ratio (mole %)*				*Guanine mononucleotide obtained by RNase T_1 digestion*		
					% of total guanylic acid		*mole % of total nucleotides*
	Guanylic	*Adenylic*	*Cytidylic*	*Uridylic*	*Calculated**	*Determined*	*Determined*
Yeast RNA (Schwarz)	29.0	25.8	20.4	24.8	29.0	50.5	14.6
Yeast RNA-9	27.5	25.6	20.5	26.4	27.5	48.7	12.7
TMV-RNA	24.0	28.0	19.2	28.8	24.0	55.7	13.4

* On basis of random distribution.

The results are shown in Table I. The amount of guanine mononucleotide in the RNase T_1 digest corresponds to the amount of guanylic acid clusters containing

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Abbreviations: RNA, ribonucleic acid; TMV, tobacco mosaic virus; Tris, tris(hydroxymethyl) aminomethane.

two or more guanylic acid in RNA. In TMV-RNA, this value is larger than in yeast RNA, and both deviate significantly from the random distribution. In these RNA's guanine nucleotide tends to be next to guanine nucleotide. The RNase T_1 attacked the highly polymerized RNA as well as low-molecular-weight RNA.

Further studies on the nucleotide distribution in RNA with the combination of the use of RNase T_1, pancreatic RNase I, and other methods are now in progress.

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1 K. SATO AND F. EGAMI, *J. Biochem.* (*Tokyo*), 44 (1957) 753; K. SATO-ASANO, *J. Biochem.* (*Tokyo*), 46 (1959) 31.
2 K. SATO-ASANO AND F. EGAMI, *Nature*, 185 (1960) 462.
3 A. GIERER AND G. SCHRAMM, *Z. Naturforsch.*, 11b (1956) 138.
4 K. MIURA *et al.*, manuscript in preparation.
5 A. M. CRESTFIELD, K. C. SMITH AND F. W. ALLEN, *J. Biol. Chem.*, 216 (1955) 185.
6 K. MIURA, T. KITAMURA AND Y. KAWADE, *Biochim. Biophys. Acta*, 27 (1958) 421.
7 R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 49 (1951) 401.
8 K. MIURA AND K. SUZUKI, *Biochim. Biophys. Acta*, 22 (1956) 565.

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Isolation of an enzyme catalyzing the transfer of amino acids from soluble RNA to microsomal ribonucleoprotein

In the course of incorporation of amino acids into microsomal RNP, recent investigations have demonstrated that activated amino acids formed a complex with a soluble RNA of relatively low molecular weight, and that the amino acids so bound to s-RNA were subsequently transferred to RNP[1,2]. Although it has been assumed that the latter transfer reaction is catalyzed by an enzyme which might be called "transferring enzyme" present in the cytoplasmic supernatant[2,3], isolation of such an enzyme has not yet been accomplished.

An approx. 15 % rat-liver homogenate was prepared in 0.25 *M* sucrose, 0.025 *M* KCl, 0.005 *M* $MgCl_2$, and 0.05 *M* Tris buffer, pH 7.6, and was centrifuged for 2 h at 105,000 × *g*. The supernatant fluid thus obtained was removed and its pH was lowered to 4.8–5.0 by 1 *N* acetic acid. The resulting precipitate was removed by a low-speed centrifugation (pH-5 fraction), and the pH of the supernatant was immediately adjusted again to pH 7.6 (pH-5 supernatant). Since the pH-5 supernatant showed a higher transferring activity than the pH-5 fraction and it does not catalyse activation of amino acids or overall incorporation of amino acids into RNP[2–4], the pH-5 supernatant was used for the isolation of the transferring enzyme. The method of purification of RNP from the microsome was the same as described in the preceding paper[5],

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Abbreviations: RNA, ribonucleic acid; s-RNA, soluble RNA; RNP, ribonucleoprotein; GTP, ATP, CTP, and UTP, triphosphates of guanosine, adenosine, cytidine, and uridine; Tris, tris-(hydroxymethyl)aminomethane; DEAE-cellulose, diethyaminoethyl-cellulose.

and s-RNA–[^{14}C]leucine was prepared from the pH-5 fraction by the method of HOAGLAND *et al.*[1].

The pH-5 supernatant, without dialysis, was poured onto an DEAE-cellulose column, 1 cm in diameter and 8 cm in height, and the column was washed with 0.03 *M* KCl containing 0.02 *M* Tris buffer, pH 7.6. Approx. 20 % of the protein was adsorbed on the column, forming a yellow band, while the bulk of the protein contained in the original pH-5 supernatant passed through the column. The adsorbed protein can be eluted from the column with a concentration of KCl between 0.08 and 0.3 *M*. In a preliminary experiment, the adsorbed protein was eluted in two steps by using 0.15 *M* KCl and 0.3 *M* KCl each containing 0.02 *M* Tris buffer, pH 7.6, after washing the column with 0.08 *M* KCl–0.02 *M* Tris buffer, pH 7.6.

Transferring activity was observed only with the protein adsorbed on DEAE-cellulose, and was highest in the fraction eluted between 0.15–0.3 *M* KCl, as shown in Table I. The active fraction gave an absorption maximum at 280 mμ and a minimum at 250 mμ, showing a typical spectrum of a protein. When the fraction was boiled and added instead of the "native" fraction, no transfer occurred. The enzyme preparation has a considerable stability and could be kept at least one week in a refrigerator without loss of activity, whereas the original pH-5 supernatant quickly lost its activity. It was also found that the enzyme fraction does not catalyse the incorporation of free [^{14}C]leucine into RNP or s-RNA.

TABLE I

FRACTIONATION OF THE pH-5 SUPERNATANT FROM RAT-LIVER CYTOPLASM BY DEAE-CELLULOSE COLUMN

Incubation mixture contained, in a final volume of 1.0 ml per tube, 2.5 mg purified RNP; s-RNA–[^{14}C]leucine, 180 counts/min (A_{260} = 1.6); 0.5 μmole ATP; 0.5 μmole GTP; 10 μmoles phosphocreatine, 0.05 mg creatine kinase; 5 μmoles $MgCl_2$; 30 μmoles KCl; 50 μmoles Tris buffer, pH 7.6. 0.1 ml of each fraction was added and the mixture incubated for 15 min at 37°. The reaction was stopped by 0.5 *N* $HClO_4$, washed once with cold 0.5 *N* $HClO_4$, and then the RNA was hydrolyzed by incubating for 15 min at 70° in 0.5 *N* $HClO_4$. The remaining protein fraction was washed twice with cold 0.2 *N* $HClO_4$ and mounted on a filter paper. The ^{14}C activity was counted by a low-background gas-flow counter (background count lower than 1 count/min).

Fractions	*Protein (mg)*	*Activity transferred (counts/min)*	*Protein recovery (mg)*
Whole pH-5 supernatant	0.73	18	
	2.90	92	279
DEAE-cellulose			
non-adsorbed fraction	0.60	9	210
0.08–0.15 *M* KCl eluate	0.61	72	24
0.15–0.3 *M* KCl eluate	0.55	94	17

When the effect of the other cofactors on the transfer reaction was studied, it was found that only GTP is essential for the transfer, and that ATP or any other cofactor tested has no effect (Table II). Addition of cell dialyzate inhibits the reaction. An observation that ATP and an ATP-generating system is essential in this transfer[3] may be accounted for the fact that the whole pH-5 supernatant was used as the enzyme fraction.

TABLE II

THE COFACTOR REQUIREMENTS FOR THE TRANSFER REACTION

Incubation mixture contained, in a final volume of 1.0 ml per tube, 2.5 mg RNP; s-RNA–[^{14}C]leucine 180 counts/min ($A_{260} = 1.6$); 0.55 mg enzyme fraction; 5 μmoles $MgCl_2$; 30 μmoles KCl; 50 μmoles Tris buffer, pH 7.6. After incubation for 15 min at 37°, the reaction was stopped by 0.5 N $HClO_4$ and counts in protein were determined according to the method described in Table I. ATP, GTP, UTP, and phosphocreatine were supplied by the Sigma Chemical Co.

Incubation conditions	*Activity transferred (counts/min)*
Incubation mixture	0
+ 0.3 μmole GTP	97
+ 0.3 μmole ATP	6
+ 0.3 μmole CTP	7
+ 0.3 μmole UTP	5
+ GTP, ATP, CTP, UTP, each 0.3 μmole	104
+ 0.3 μmole GTP, 10 μmoles phosphocreatine, 0.05 mg creatine kinase	102
+ 0.3 μmole GTP, 0.3 μmole ATP, 10 μmoles phosphocreatine, 0.05 mg creatine kinase	98
+ 0.3 μmole GTP, cell dialyzate	75

Further purification and characterization of the transferring enzyme are in progress.

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[1] M. B. HOAGLAND, M. L. STEPHENSON, J. F. SCOTT, L. I. HECHT AND P. C. ZAMECNIK, *J. Biol. Chem.*, 231 (1958) 241.
[2] P. C. ZAMECNIK, M. L. STEPHENSON AND L. I. HECHT, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 73.
[3] L. G. GROSSI AND K. MOLDAVE, *Biochim. Biophys. Acta*, 35 (1959) 275.
[4] M. TAKANAMI, to be published.
[5] M. TAKANAMI, *Biochim. Biophys. Acta*, 39 (1960) 318.

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Isolation of free acid-soluble nucleotide peptides from normal rat liver

It has been shown by WEINSTEIN *et al.*[1] that normal rat liver contains nucleotides of adenine, cytosine and uracil associated with amino acids or peptides. WILKEN AND HANSEN[2] also identified two adenine nucleotide peptides from bovine liver.

During our investigation of nucleotide peptides from normal rat liver we have been able to isolate three different peptides associated with an adenine-free acid-soluble nucleotide.

25 Wistar rats fed on stock laboratory diet have been utilised for this purpose. The animals, divided in groups A, B and C, were killed by exsanguination, the livers

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Abbreviations: ADP, adenosine 5′-diphosphate; UDPAG, uridine 5′-diphosphate acetyl glucosamine; UDPG, uridine 5′-diphosphate glucose; DPN, diphosphopyridine nucleotide; UMP, uridine 5′-monophosphate; CMP, cytidine 5′-monophosphate; and TMP, thymidine 5′-monophosphate.

quickly removed and extracted by the cold-$HClO_4$ method previously described by ONDARZA[3,4]. The extracts were applied to columns of Dowex 1, chloride form (20 × 1.2 cm) according to the method of COHN[5], the nucleotides eluted with 0.01 N HCl in a gradient concentration of NaCl, and recovered from the eluates by charcoal adsorption and elution as described by SMITH AND MILLS[6].

Between the compounds ADP and UDPAG eluted with the solvents 0.01 N HCl–0.01 M NaCl and 0.01 N HCl–0.03 M NaCl appeared from rat-liver group A two ultraviolet-absorbing spots, named compounds I and II, and another two spots from groups B and C corresponding to compounds I and III (see Table I).

TABLE I

Nucleotide peptide	R_{UDPG}*	R_{DPN}*
Compound I	0.41	0.64
Compound II	0.27	0.40
Compound III	0.26	0.40

* Obtained by descending paper chromatography with the ethanol–ammonium acetate solvent of PALADINI AND LELOIR[7].

The above separated nucleotides were eluted with water after washing the paper with absolute alcohol, and the following analysis carried out, mainly with compound III.

(a) 0.2 µmole of compounds I, II and III were hydrolysed with 6 N HCl (110° for 18 h) in a sealed tube, evaporated until free of acid and then chromatographed bidimensionally with the solvents butanol–acetic acid–water (4:1:5) and phenol–water (75:25). The chromatograms after reaction with the ninhydrin reagent in isopropanol are shown in Fig. 1.

(b) Hydrolysis of 0.3 µmole of compound III with 2 N HCl (100° for 1 h), followed by evaporation to dryness and separation by paper chromatography for bases by the method of WYATT[8], gives a visible spot with the u.v. lamp. The R_F value of this spot is shown in Table II along the values for the standards.

The spot from compound III after elution with 0.1 N HCl gives a maximum absorption at 262 mµ; at pH 9, this is at 267 mµ.

(c) Estimations of base by absorption at 262 mµ at pH 2 (assuming a value of 11,500 for the molar extinction coefficient), total phosphorus[9] and sugar by the orcinol

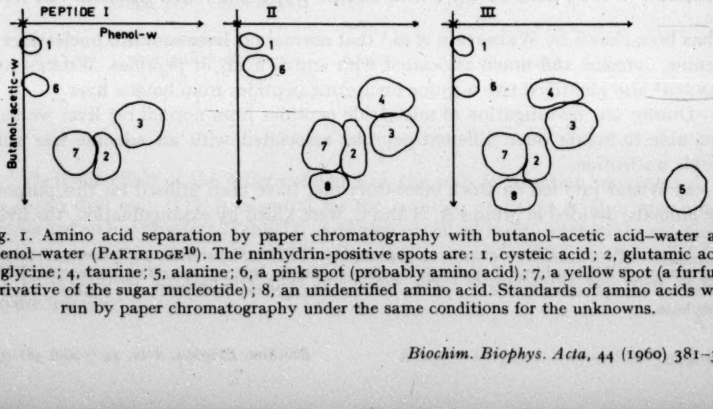

Fig. 1. Amino acid separation by paper chromatography with butanol–acetic acid–water and phenol–water (PARTRIDGE[10]). The ninhydrin-positive spots are: 1, cysteic acid; 2, glutamic acid; 3, glycine; 4, taurine; 5, alanine; 6, a pink spot (probably amino acid); 7, a yellow spot (a furfural derivative of the sugar nucleotide); 8, an unidentified amino acid. Standards of amino acids were run by paper chromatography under the same conditions for the unknowns.

method[11] using *d*-ribose as standard, give the ratios: base/sugar/phosphorus = 1:0.99:0.96.

TABLE II

Bases and nucleotides	*RF in HCl-Isopropanol*[8]
Guanine	0.35
Hypoxanthine	0.40
Adenine	0.55
Compound III	0.55
CMP	0.61
UMP	0.68
TMP	0.80

It is concluded that the material isolated is an adenine ribonucleoside-monophosphate peptide. The peptide, as far as we know, has not previously been described in this form.

It is possible that these nucleotide peptides are involved in protein synthesis, as has been postulated by HOAGLAND[12] for active amino acids. If this is the case, these peptides must be in an active form when bound to a nucleotide, and should be considered as "activated" peptides.

The sequence of the amino acids and the type of union between the nucleotide and the peptide is under investigation in this laboratory.

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[1] C. WEINSTEIN, D. HAMMOND, J. I. BERKMAN, P. M. GALLOP AND S. SEIFTER, *Federation Proc.*, 17 (1958) 332.
[2] D. R. WILKEN AND R. G. HANSEN, *Federation Proc.*, 19 (1960) 309.
[3] R. N. ONDARZA, *Acta Physiol. Latinoam.*, 7 (1957) 147.
[4] R. N. ONDARZA, *Acta Physiol. Latinoam.*, (1960) in the press.
[5] W. E. COHN, *J. Am. Chem. Soc.*, 72 (1950) 1471.
[6] E. E. B. SMITH AND G. T. MILLS, *Biochim. Biophys. Acta*, 13 (1954) 587.
[7] A. C. PALADINI AND L. F. LELOIR, *Biochem. J.*, 51 (1952) 426.
[8] G. R. WYATT, *Biochem. J.*, 48 (1951) 584.
[9] L. F. LELOIR AND C. E. CARDINI, *Methods in Enzymology*, vol. III (1957) 840.
[10] S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238.
[11] H. G. ALBAUM AND W. W. UMBREIT, *J. Biol. Chem.*, 167 (1947) 369.
[12] M. B. HOAGLAND, *Biochim. Biophys. Acta*, 16 (1955) 288.

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The amino acid sequence of peptide B of co-fibrin

Evidence obtained from peptide fragments identified in pepsin digests of desulfated Peptide B is presented here in support of a proposed complete amino acid sequence for this peptide.

Peptide B is one of the two large acidic peptides released from the N-terminal portion of bovine fibrinogen through the hydrolytic action of bovine thrombin[1]. The complete amino acid sequence of the other acidic peptide, Peptide A, has been

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Proposed sequence: N-acetyl–Thr–Glu–Phe–Pro–Asp–Tyr(SO_4)–Asp–Glu–Gly–Glu–Asp–Asp–Arg–Pro–Lys–Val–Gly–Leu–Gly–Ala–Arg

Peptide fragments identified in peptic digests

N-acetyl–Thr–Glu
(Thr–Glu, Phe, Pro, Asp)Tyr
Phe
Pro–Asp–Tyr
Pro–Asp–Tyr(Asp, Glu)
Tyr–Asp–Glu
Tyr–Asp–Glu–Gly(Glu, Asp)
Tyr–Asp–Glu–Gly(Glu, Asp, Asp, Arg, Pro, Lys, Val, Gly)Leu
Asp–Glu
Gly–Glu–Asp
Gly–Glu–Asp–Asp(Arg, Pro, Lys, Val, Gly)Leu
Asp–Arg–Pro–Lys–Val–Gly–Leu
Gly–Ala–Arg

Fragments in chymotryptic digests[3]

(Thr, Phe, Tyr, Glu_3, Asp_4, Arg, Pro_2, Lys, Val, Gly_2)Leu
Gly–Ala–Arg

Fragments in tryptic digests[3]

(Thr, Phe, Tyr, Glu_3, Asp_4, Arg, Pro_2, Gly, Lys)
Val–Gly–Leu–Gly–Ala–Arg

Fig. 1. Summary of data leading to the proposed amino acid sequence of Peptide B.

reported[2]. A partial sequence of Peptide B was formulated from fragments obtained following digestion by trypsin and chymotrypsin[3]. Recently BLOMBÄCK AND SJÖQUIST[4] have reported the location of several more amino acid residues in this peptide.

The proposed amino acid sequence of Peptide B is presented in Fig. 1 along with a listing of peptide fragments which have been identified in peptic digests of the desulfated peptide.

The sulfate was quantitatively removed from the tyrosine sulfate residue of peptide B[5] without cleavage of peptide bonds by treatment with 1 *N* HCl at 25° for 20 h[6]. Peptic digestion was carried out at 37° in 0.01 *N* HCl with a 1:150 molar ratio of enzyme to substrate. Peptide fragments were separated and purified by chromatography on Dowex 50,X2 followed by paper chromatography or paper electrophoresis.

The peptide fragment, which appears to be N-acetyl-Thr-Glu, was isolated and identified by methods similar to those employed by NARITA for studies of the N-acetyl dipeptide from digests of tobacco-mosaic-virus protein[7]. A 72-h peptic digest of Peptide B was passed into a column of Dowex 50 (X2, H^+ form). The ninhydrin-negative fragment was detected by chromatographic analysis of acid-hydrolyzed portions of water-effluent fractions; equimolar quantities of glutamic acid and threonine were found in the hydrolyzates. Treatment of the fragment with high concentrations of carboxypeptidase A for long periods resulted in the release of small amounts of glutamic acid. Chromatography of a hydrazinolyzate of this ninhydrin-negative peptide fragment, employing the pyridine–aniline–water and collidine–water systems of NARITA[7], led to the identification of free glutamic acid (R_F, 0.30 and 0.0 in the two systems, respectively) and threonine hydrazide (R_F, 0.55 and 0.15, respectively). A compound having the R_F of acetyl hydrazide was observed in each chromatographic system in approximately equimolar concentration to threonine hydrazide.

N-acetylseryltyrosine has been identified in the α-melanocyte-stimulating hormone of pig pituitary[8] as well as in tobacco-mosaic-virus protein[7] as the N-terminal sequence. The present work strongly supports the existence of an N-acetyl blocking group at the N-terminus of Peptide B, and hence at one of the N-termini of bovine fibrinogen. Furthermore, the presence of this grouping explains the previous failure to find an N-terminal residue in Peptide B by usual methods of N-terminal analysis[1,3,4].

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[1] K. BAILEY, F. R. BETHELHEIM, L. LORAND AND W. R. MIDDLEBROOK, *Nature*, 167 (1951) 223.
[2] J. E. FOLK, J. A. GLADNER AND Y. LEVIN, *J. Biol. Chem.*, 234 (1959) 2317.
[3] J. E. FOLK, J. A. GLADNER AND K. LAKI, *J. Biol. Chem.*, 234 (1959) 67.
[4] B. BLOMBÄCK AND J. SJÖQUIST, *Acta Chem. Scand.*, 14 (1960) 493.
[5] F. R. BETTELHEIM, *J. Am. Chem. Soc.*, 76 (1954) 2838.
[6] J. E. FOLK, J. A. GLADNER AND K. LAKI, *Federation Proc.*, 18 (1959) 228.
[7] K. NARITA, *Biochim. Biophys. Acta*, 28 (1958) 184.
[8] J. I. HARRIS, *Biochem. J.*, 71 (1959) 451.

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The hydrolysis of dipeptides by rat intestinal extracts

The capacity of rat intestine to hydrolyse the dipeptides glycylglycine, glycyl-L-leucine, L-leucylglycine and glycyl-L-alanine, is greatest in the ileum and decreases towards the duodenum[1]. This work has been extended to include the colon as well as the small intestine; the dipeptides used were glycyl-L-valine and glycylglycine.

Intestines from adult male rats, Wistar strain, were used. The rats had been starved for 16 h before death to ensure an empty intestine. Mucosal extracts were prepared at 4° by scraping the mucosa from the central portions of duodenum, jejunum, ileum and colon. The scrapings were homogenised in 40–50 vol. of distilled water, with a homogeniser of the Potter–Elvehjem type. After allowing the homogenates to stand at 0° for 2 h, they were centrifuged at 26,000 × *g* for 20 min in a refrigerated centrifuge and the supernatants used as the source of enzyme.

Enzyme activity was assayed by incubating 0.08 ml 0.0625 *M* dipeptide with 0.08 ml of 0.2 *M* Tris buffer at an appropriate pH, and 0.04 ml of enzyme extract, at 37°. Duplicate aliquots of 0.01 ml were removed from the reaction mixture at intervals during a 1-h period and added to 1-ml samples of ninhydrin reagent[2]. These were heated at 100° for 25 min, and the intensity of the colour developed was related to the degree of hydrolysis using a standard graph. The graph was obtained using artificial hydrolysis mixtures and the same method.

The hydrolysis of glycyl-L-valine was measured at the optimal pH value of 8.4. The reaction followed first-order kinetics at the beginning of the incubation period, but the activity of the enzyme slowly declined. When a graph of $\log \frac{s}{s-x}$ (s = initial substrate concentration, x = substrate concentration at time t during the reaction) against t was plotted, a curve was obtained. The first-order rate constant for the reaction was calculated from the tangent to this curve, taken from the origin. Using this method the reaction rate was proportional to enzyme concentration.

Glycylglycine hydrolysis was found to have two pH optima, at pH 7.4 and 8.2 [1]. Determinations were carried out at pH 7.4, in the presence of 0.001 *M* Co^{++}. The reaction had zero-order characteristics and the rate of reaction was proportional to enzyme concentration.

Blanks using boiled enzyme and dipeptide or enzyme alone showed negligible increases in colour.

The nitrogen content of the extracts was determined, using the micro-Kjeldahl technique.

The distribution of the hydrolytic activities in the different intestinal regions is given in Table I. For both dipeptides the specific activity of the ileum is significantly greater than that of the duodenum or jejunum, and that of duodenum is smaller than that of jejunum. The specific activity of the colon is much lower than those of the small intestine. Variation in the specific activities of the small intestine is not explained by excess inactive protein diluting the activity of the extracts from the upper intestine. Measurements of total protein obtained from 2.5-cm segments of intestine from each region showed no significant differences[1].

The low activity found in the colon indicates that the high level of peptidase activity in the small intestine is not common to the whole intestinal tract. Presumably

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Abbreviations: Tris, tris(hydroxymethyl)aminomethane.

TABLE I

THE DISTRIBUTION OF PEPTIDE-SPLITTING ACTIVITY IN RAT INTESTINAL TRACT

(1 unit of activity = hydrolysis of 1 μmole substrate in 10 min)

	Expt.	*Specific activity (units/mg protein)*			
		Duodenum	*Jejunum*	*Ileum*	*Colon*
Gly-Val	1	39.5	42.5	64.5	8.4
	2	39.2	35.2	56.8	2.4
	3	49.5	51.5	62.0	9.9
	4	43.5	43.0	57.5	15.6
	Mean	42.9	43.5	60.2	9.1
	$P(D-J) > 0.1$		$P(J-I) < 0.02$		$P(D-I) < 0.01$
Gly-Gly	1	1.8	5.3	7.4	1.7
	2	3.6	5.6	6.6	1.4
	3	6.2	6.7	9.8	1.9
	4	7.1	6.8	10.1	—
	5	8.2	6.7	11.3	3.2
	Mean	5.4	6.2	9.0	2.0
	$P(D-J) > 0.1$		$P(J-I) < 0.05$		$P(D-I) < 0.02$

the high activity of the small intestine is related to its absorptive function. NEWEY AND SMYTH[3] have shown that intact dipeptides enter the mucosal cells *in vitro*, but that the dipeptides are almost completely hydrolysed before they appear on the serosal side of the intestine[4]. This finding is in agreement with the postulate of FLOREY, WRIGHT AND JENNINGS[5] that the function of intestinal peptidases is primarily intracellular.

In view of the high peptidase level in the ileum, it seems likely that absorption of dipeptides and their intracellular hydrolysis may be maximal in this region of the intestinal tract. A high absorptive capacity of the lower small intestine could have two physiological functions. Either maximum absorption occurs in this region, or maximum absorption occurs in the upper intestine, and the high activity in the lower intestine would then make certain of the absorption of remaining traces of food from the lumen, resulting in a highly efficient digestive process.

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[1] G. B. ROBINSON AND B. SHAW, *Biochem. J.*, in the press.

[2] S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 176 (1948) 367.

[3] H. NEWEY AND D. H. SMYTH, *J. Physiol. (London)*, 152 (1960) 367.

[4] H. NEWEY AND D. H. SMYTH, *J. Physiol. (London)*, 145 (1959) 48.

[5] H. W. FLOREY, R. D. WRIGHT AND M. A. JENNINGS, *Physiol. Revs.*, 21 (1941) 36.

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Biological and chemical properties of α-amylase precursor

Previous studies[1] in this laboratory suggest that the last process of α-amylase formation in *Bacillus subtilis* occurs by a transformation of a previously formed precursor protein. It was shown that about 7 % of total cellular protein was transformed into α-amylase and excreted into the medium after the cell growth reached the stationary phase.

B. subtilis (strain K) was grown on the surface of the synthetic medium as described in a previous paper[1]. Sonic extracts of the bacterial cells were centrifuged and the supernatant was fractionated by column chromatography using DEAE-cellulose.

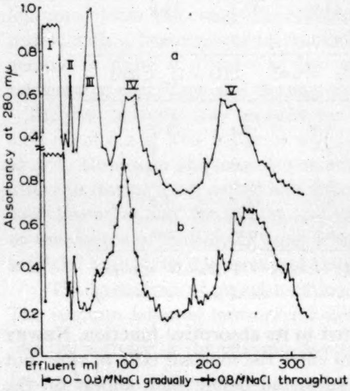

Fig. 1. Elution pattern of bacterial extract of *B. subtilis*. The bacterial cells were disintegrated by sonic oscillation (Tominaga Works, Japan, 8 kc, approx. 100 W, 30 min) in 0.02 *M* tris(hydroxymethyl)aminomethane buffer, pH 8.0, and centrifuged for 30 min at 10,000 rev./min in a Servall (SS-1) centrifuge. An aliquot of the supernatant (approx. 100 mg dry weight) was placed on a DEAE-cellulose column (0.9 × 25 cm), and eluted with a linear salt gradient at 13 ± 1°.

Typical elution patterns are shown in Figs. 1a and 1b. Five distinct protein peaks were obtained from the cells of the earlier stage at which the cell growth almost reached the stationary phase, but α-amylase activity in the medium was only 300 $D^{30'}_{40^\circ}$/ml in Wohlgemuth's units[2] (Fig. 1a). On the other hand, the peak marked III could not be recognized in the extract of the cells at the latter stage of growth, (Fig. 1b). At this stage, α-amylase activity in the medium was 3,500–4,000 $D^{30'}_{40^\circ}$/ml, *i.e.* α-amylase production was almost complete. The amount of the protein III which disappeared during this period was comparable to the amount of α-amylase produced in the medium. These results, together with the fact that the cells at an intermediate stage of α-amylase production contained a moderate amount of the protein III, suggest that the latter may be an α-amylase precursor. To substantiate this possibility, immunological and chemical methods were employed.

Rabbit anti-α-amylase serum was prepared by a series of intravenous injections of crystalline α-amylase, giving a total of 80 mg per rabbit. Antigenic homogeneity was established by the formation of a single line precipitation in gel diffusion agar with anti-serum and α-amylase. The ratio between α-amylase and anti-serum is about 1:3 at the equivalence point.

Antigenicity of the protein fractions obtained by column chromatography was tested by precipitation reactions and by their activity as an inhibitor of the α-amylase-anti-sera neutralization reaction under various conditions. The protein III gave

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Abbreviation: DEAE-, diethylaminoethyl-.

positive results in both tests, and none of the other protein fractions had any cross reaction.

Gel-diffusion precipitation between anti-sera, α-amylase and the protein III showed a continuous line (Fig. 2), which indicates that the protein III is related antigenically to α-amylase.

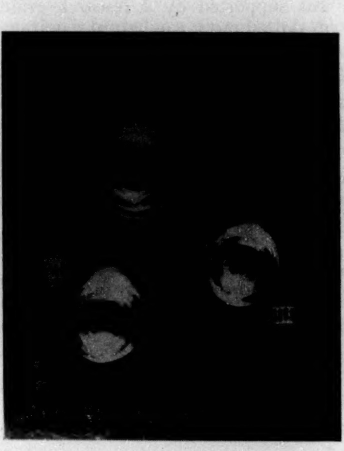

Fig. 2. Gel-diffusion-precipitation reaction between anti-serum, α-amylase and the protein III
I: anti-serum, II: α-amylase, III: protein III.

Elution patterns of the extract from an α-amylase-negative mutant of *B. subtilis* were not radically different from that of the wild strain shown in Fig. 1a. However, none of the protein classes had any cross reaction with the anti-serum.

The protein III was lyophylized after dialysis, and some of its chemical properties were investigated. It had very weak α-amylase activity, *i.e.* 1.5–2 % that of α-amylase, and after one rechromatography, about 0.6 %. This activity probably comes from a small amount of α-amylase contaminating the protein III.

Judging from the following results, the protein III is fairly homogeneous: (1) it showed a single elution peak by rechromatography, (2) it showed almost homogeneous sedimentation pattern by ultra-centrifugation, (3) it gave a single N-terminal amino acid, *i.e.* phenylalanine, by the dinitrofluorobenzene method.

From the sedimentation coefficient of the protein III ($S_{20,w} = 3.88$ s), together with the known sedimentation coefficient (4.22 s) and molecular weight (49,000) of α-amylase[3], the molecular weight of the protein III may be estimated tentatively as about 43,000.

If the protein III is an α-amylase precursor, both proteins should have common peptide chains in their molecules. "Peptide maps" of the tryptic hydrolysate of both proteins obtained by the method described by KATZ *et al.*[4] were compared. α-Amylase gave about 40 peptide spots and the protein III gave about 20. Among these spots, at least ten might be considered as common peptides. The amino acid compositions of the peptides are now being elucidated.

The protein III contains much more glutamic acid, probably in amide form, and a lesser amount of arginine than α-amylase.

The results described above and the results presented in a previous paper[1] strongly suggest that the protein III is not merely "cross-reacting material"[5–7], but it must be a real α-amylase precursor.

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[1] A. YOSHIDA AND T. TOBITA, *Biochim. Biophys. Acta*, 37 (1959) 513.
[2] J. WOHLGEMUTH, *Biochem. Z.*, 6 (1908) 1.
[3] A. YOSHIDA, *Biochim. Biophys. Acta*, 41 (1960) 98.
[4] A. M. KATZ, W. J. DREYER AND C. B. ANFINSEN, *J. Biol. Chem.*, 234 (1959) 2897.
[5] M. COHN AND A. M. TORRIANIS, *J. Immunol.*, 69 (1952) 471.
[6] S. R. SUSKIND, *J. Bacteriol.*, 74 (1957) 308.
[7] P. LERNER AND C. YANOFSKY, *J. Bacteriol.*, 74 (1957) 494.

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The use of urea-starch-gel electrophoresis in studies of reductive cleavage of an α_2-macroglobulin

The presence of several protein components of high molecular weight in human serum is well established[1]. There are two major groups of proteins with sedimentation coefficients between 17 S–20 S. One of these groups is comprised of proteins belonging to the γ_1-globulins[2,3]; the other is composed mainly of proteins belonging to the α_2-globulins[4]. It has been postulated that these macromolecules represent polymeric proteins[5,6]. DEUTCH AND MORTON[7] have shown that the addition of mercaptoethanol and cysteine result in a fall in sedimentation rate from 18 S to 7 S and that stable monomeric units are formed in the presence of iodoacetamide.

This report is concerned with the effects of reductive cleavage with mercaptoethanol in urea on the protein of high molecular weight belonging to the α_2-globulins. This protein is variously known as 'heat-labile glycoprotein"[8], the "alpha 2 macroglobulin"[4], and as "slow alpha 2 globulin"[9,10]. SCHONENBERGER *et al.*[11] studied the physicochemical properties of this protein in great detail and described dissociation of the protein into two components (11 S and 15.7 S) in the presence of 5 M urea. Reaggregation to a single component of 18.1 S occurred after removal of the urea with and without the presence of iodoacetamide. Comparable results were obtained by ISLIKER[12], who measured the change in viscosity of the protein after the addition of cysteine, cysteamine and thioglycollate. Ultracentrifugal analysis revealed a 6 S component after treatment of the proteins with these components.

A method is described using starch-gel electrophoresis for the separation and demonstration of products obtained by reductive cleavage with mercaptoethanol in urea and after stabilization of the –SH groups by addition of iodacetamide. Gels for this purpose are conveniently prepared by mixing 60–65 g of Starch Hydrolysed (Connaught Medical Research Laboratories, Toronto, Canada) and 240 g of urea. Small portions of the mixture are then rapidly poured into a flask (2000 ml) containing 300 ml of buffer and vigorously stirred by hand between each addition.

The highly viscous solution is heated in a water bath (70°) with occasional stirring for 10 min. After removal of the trapped air, the gel is poured into a tray, with occasional stirring for 10 min, similar to that used for vertical gel electrophoresis[13] and allowed to settle for 24 h.

A preparation of the α_2-macroglobulin (generous gift of Dr. H. E. SCHULTZE, Behringwerke, Germany) was used in all experiments. Starch-gel electrophoresis in the discontinuous system of buffers[14] disclosed that this preparation contained one major band and a minor less rapidly migrating component. An 0.5 % solution of the protein was prepared in: (a) 0.1 M borate buffer, (b) 8 M urea (recrystallized) and 0.1 M borate buffer, (c) the same as (b) with the addition of mercaptoethanol, final concn. 0.02 M, (d) the same as (c) with the addition of iodoacetamide, final concn. 0.05 M. The solutions, after standing for 2–3 h at room temperature, were then subjected simultaneously to electrophoresis for 24 h at a voltage gradient of 6 V/cm in starch gels prepared in: (a) borate buffer[9], (b) borate buffer and 8 M urea[15], (c) formate buffer (0.05 M formic acid, 0.01 M NaOH; pH3.1) and 8 M urea (SMITHIES, personal communication). After completion of the experiment the gel was stained in the usual manner with Amido Black 10B. The greatest separation was achieved in the formate urea gel as shown in Fig. 1.

Five heavy and two faint bands can be observed in the control (S α_2 dissolved in borate buffer alone) as well as in borate buffer and 8 M urea (Fig. 1A and B). The two patterns are similar though pattern 1B shows clearer separation of the bands, probably due to the equilibrium achieved between the urea in the gel and that of the applied material.

Mercaptoethanol-treated material separated into four main bands as shown on Fig. 1C. However, some of the protein did not migrate into the gel as judged by the amount of stained material left at the origin (visible as heavy line on the photograph). The iodoacetamide-treated material was also separated into four bands (Fig. 1D) and four additional minor bands were seen though they are not readily shown by photography. If the reactions are allowed to proceed at 10° for 24 h a similar pattern is obtained with additional separation of the slowest component of the iodoacetamide-treated material. In contrast to the results obtained using mercaptoethanol alone (1C), complete entry of the protein into the gel occurred when iodoacetamide was added (1D). The findings suggest the probability that slow α_2-globulin contains subunits linked, at least in part, by disulfide bonds. Other linkages, however, cannot be excluded[11]. Ultracentrifugal studies on the isolated components are in progress. It was observed that the separated components exhibit a much greater avidity for the Amido Black dye than the original material, owing to the additional groups made accessible to the dye by the opening of side chains. These results indicate that the subunits of the slow α_2-globulin may be successfully separated using the technique of urea-starch-gel electrophoresis. Application of this method appears useful in structural studies on a variety of purified proteins.

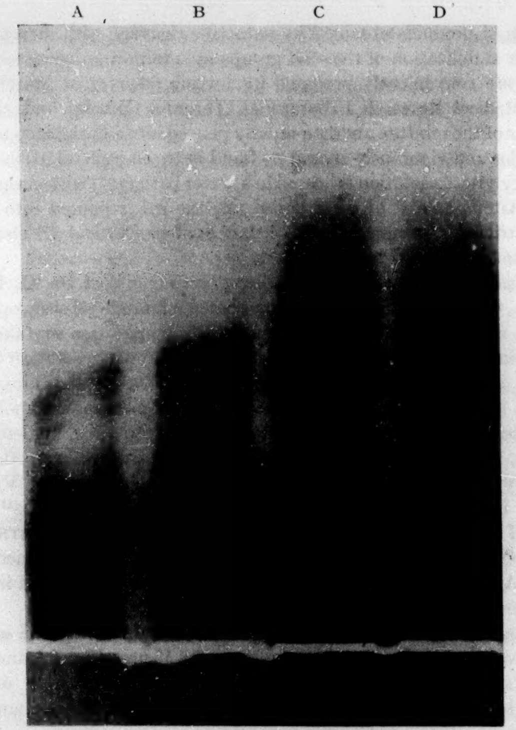

Fig. 1. Vertical urea-starch-gel electrophoresis of S α_2-globulin after reductive cleavage. Starch gel is prepared in 8 M urea and formate buffer (0.05 M formic acid, 0.01 M NaOH; pH 3.1) and electrophoresis conducted at 6 V/cm for 24 h. A. Slow α_2, borate buffer. B. Slow α_2, borate and urea. C. Slow α_2, borate, urea and mercaptoethanol. D. Slow α_2, borate, urea, mercaptoethanol and iodoacetamide.

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[1] H. G. KUNKEL, in *The Plasma Proteins*, Associated Press, New York, 1960, p. 279.
[2] H. I. MÜLLER-EBERHARD, H. G. KUNKEL AND E. C. FRANKLIN, *Proc. Soc. Exptl. Biol. Med.*, 93 (1956) 146.
[3] G. WALLENIUS, R. TRAUTMAN, H. G. KUNKEL AND E. C. FRANKLIN, *J. Biol. Chem.*, 225 (1957) 253.
[4] H. E. SCHULTZE, I. S. GÖLLNER, K. HEIDE, M. SCHONENBERGER AND G. SCHWICK, *Z. Naturforsch.*, 10b (1955) 463.
[5] K. O. PEDERSEN, *Ultracentrifugal Studies on Serum and Serum Fractions*, Almquist and Wiksells, Uppsala, 1945, p. 162.
[6] E. C. FRANKLIN AND H. G. KUNKEL, *J. Immunol.*, 78 (1957) 11.

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[7] H. F. Deutch and J. I. Morton, *J. Biol. Chem.*, 231 (1958) 1107.

[8] R. K. Brown, W. H. Baker, A. Peterkovsky and D. L. Kauffman, *J. Am. Chem. Soc.* 76 (1954) 4244.

[9] O. Smithies, *Biochem. J.*, 61 (1955) 629.

[10] M. D. Poulik and O. Smithies, *Biochem. J.*, 68 (1958) 636.

[11] M. Schonenberger, R. Schmidtberger and H. E. Schultze, *Z. Naturforsch.*, 13b (1958) 761.

[12] H. Isliker, *Helv. Med. Acta*, 25 (1958) 41.

[13] O. Smithies, *Biochem. J.*, 71 (1959) 585.

[14] M. D. Poulik, *Nature*, 180 (1957) 1477.

[15] O. Smithies and G. E. Connell, in *Ciba Foundation Symposium on Biochemistry of Human Genetics*, Little Brown & Company, New York, 1959, p. 178.

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Polarographic identification of the reactive form in the hydrolysis of pyridoxal 5-phosphate

The monoanion of phosphoric acid monoalkyl and monoaryl esters undergoes hydrolysis more easily than the undissociated form or the dianion[1]. In view of its biochemical importance, the hydrolysis of pyridoxal 5-phosphate was studied, using

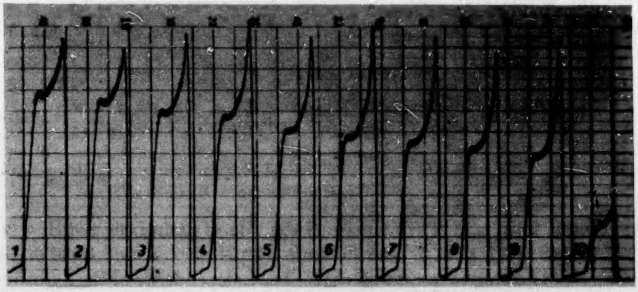

Fig. 1. Polarographic record of the course of hydrolysis of pyridoxal 5-phosphate in 0.01 M $HClO_4$ at $I = 1.0$, and at 79°: $5 \cdot 10^{-4}$ M pyridoxal 5-phosphate after: (1) 15; (2) 23; (3) 41; (4) 50.5; (5) 79; (6) 94.5; (7) 110; (8) 125 (9) 152 min. Curve 10, $5 \cdot 10^{-4}$ M pyridoxal. Curves were registered on a pen-recording polarograph, constructed by Institut für Gerätebau DAW, Berlin, D.D.R. Full scale deflection, 10 μA.

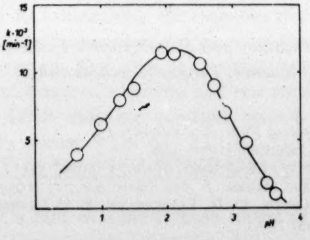

Fig. 2. The dependence of the rate constant of hydrolysis of pyridoxal 5-phosphate on pH at 87.8° and $I = 1.0$.

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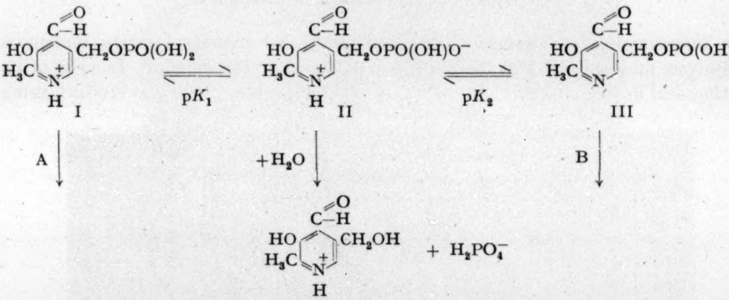
the polarographic method. In $HClO_4$ solutions and in buffers at pH 1–4 (I, 1.0) at 60–90° the decrease of the diffusion current of pyridoxal 5-phosphate was measured (Fig. 1). Pyridoxal formed in this reaction gives under these conditions a kinetic current[2,3] which represents only 10–30% of the total wave height, and does not interfere with the measurements. The pH-dependence of the first-order rate constant (Fig. 2) shows a maximum at pH 2.1. From the rising part of the curve the value of the first dissociation constant $pK_1 = 1.5$, corresponding to the dissociation of the first proton from the phosphoric acid part of the molecule, was estimated. This value is in accordance with the pK_1 for monobenzyl phosphate[4]. The declining part of the curve lies in the region of $pK_2 = 3.65$ (determined potentiometrically). This value is in the region of pK values found with other pyridine derivatives. For phosphoric acid aryl esters no pK value has been found in this region[4,5]. The pK_2 value is attributed to the dissociation of a proton from the pyridine nitrogen, thus:



(1)

The reactive form is II. The increase of the rate of hydrolysis at pH < 2 is due to the formation of II from I; the decrease at pH > 3 is due to a diminishing concentration of II owing to formation of III. The independence of the hydrolysis rate on the type and concentration of the buffer and the small increase of the rate constant wit[illegible]ncreasing ionic strength are in agreement with this scheme. Reactions A and B are unimportant under the conditions used.

Full details of this reaction, important for understanding vitamin B_6 reactions, will be given elsewhere.

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[1] C. A. Vernon, *Chem. Soc. Special Publ. No. 8* (1957) 17.
[2] J. Volke, *Z. phys. Chem.*, Sonderheft (1958) 268.
[3] O. Manoušek and P. Zuman, *J. Electroanal. Chem.*, 1 (1960) 324.
[4] J. Kumamoto and F. H. Westheimer, *J. Am. Chem. Soc.*, 77 (1955) 2515.
[5] P. W. C. Bernard, C. A. Bunton, D. R. Llewellyn, K. G. Oldham, B. L. Silver and C. A. Vernon, *Chem. & Ind.*, (1955) 760.

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The 2,4-dinitrophenol-responsive adenosinetriphosphatase of rat-liver mitochondria

Various investigators[1–3] have postulated that DNP activation of mitochondrial ATPase is a manifestation of the system of oxidative phosphorylation. It was of interest in this regard when LARDY[2] showed that desiccation of rat-liver mitochondria gave powders that contained ATPase activity. Extracts of such powders possessed ATPase activity unsedimented at 18,000 × *g* and responsive to both DNP and Mg^{++}. This report presents results of further study of the ATPase activities present in such powders.

Early in this work it was found that a significant percentage of the DNP-responsive ATPase of the powders is not sedimented in 60 min at 105,000 × *g*. Since such preparations would present advantages in purification attempts, they have been employed routinely. Extracts are prepared by homogenizing the powders in 0.25 *M* sucrose. The suspension is clarified at 105,000 × *g*, and the supernatant is lyophilized for reconstitution as needed. Phosphate formation in the presence of ATP is determined by a modified Martin and Doty procedure[4].

The original assay for DNP-responsive ATPase[2] employed 0.09 *M* KCl at pH 7.4. This work has shown that pH 8.5 is optimal for the DNP response (P_i formation in presence of DNP less P_i formation in absence of DNP). In addition, the maximum response occurs in the absence of added salts such as KCl or NaCl. In Table I the results show that added KCl diminishes the response to DNP. Similar results occur with NaCl. Presumably, any monovalent-cation requirement the enzyme has is met by the system as constituted. The item of greatest interest in Table I, however, concerns the first column of figures where no KCl was added. Under these conditions, two concentrations of DNP differing 200-fold possess approximately the same capacity to stimulate P_i release from ATP. In the original assay system[2], in the presence of 0.09 *M* KCl, $1 \cdot 10^{-4}$ to $1 \cdot 10^{-3}$ *M* DNP had been found to yield the maximal response.

TABLE I

ION INTERFERENCE IN RESPONSE OF ATPASE TO 2,4-DINITROPHENOL

Conditions: 0.06 *M* Tris buffer, pH 8.5; 0.012 *M* ATP; enzyme containing 1.3 mg protein; additions of KCl and DNP as indicated; in volume of 0.5 ml. Incubation 2 min at 31°. P_i formation was measured directly on the assay system by a modified Martin and Doty procedure[4].

	(μmoles Pi formed/mg protein) $\times 10^2$			
Added KCl (M)	—	*0.04*	*0.08*	*0.12*
—	4.2	6.5	5.1	4.3
$5 \cdot 10^{-6}$ *M* DNP	10.5	7.2	0	0
$5 \cdot 10^{-5}$ *M* DNP	7.8	7.4	1.6	0
$5 \cdot 10^{-4}$ *M* DNP	9.4	10.3	2.5	1.6
$1 \cdot 10^{-3}$ *M* DNP	11.2	14.2	6.9	5.6

These findings gave a hint of a multiphase aspect in the response to DNP and

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Abbreviations: DNP, 2,4-dinitrophenol; ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane; P_i, inorganic phosphate therefore prompted reinvestigation of the nature of ATPase response over a wide range of DNP concentrations. Fig. 1 shows extracts that of mitochondrial acetone powders in 0.25 *M* sucrose exhibit characteristic multiphasic curves of ATPase reponse to DNP. Peaks of response occur at $1 \cdot 10^{-8}$, $1 \cdot 10^{-5}$ and $1 \cdot 10^{-3}$ *M* DNP. Curves A and B were obtained with two different powders, the second of which had a lower specific activity at more dilute DNP concentrations. Similar results have been obtained with other acetone powders.

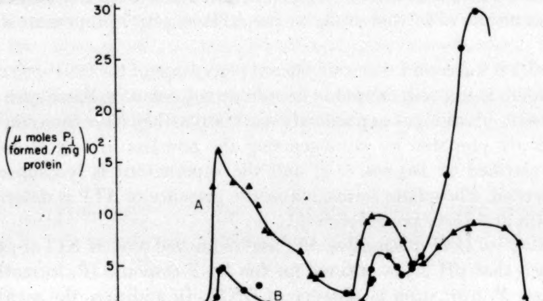

Fig. 1. Effects of variation of DNP concentration on ATPase activity. Conditions: 0.06 *M* Tris, pH 8.5; 0.012 *M* ATP; DNP in 0.5 ml. Curve A: 1.04 mg protein incubated 2 min at 28°. Curve B: 1.25 mg protein incubated 11 min at 28°.

These results may have other interpretations, but it is our feeling that they give direct evidence for the three DNP-responsive ATPases postulated by SLATER[5]. While SLATER's results indicated the presence of three such enzymes with pH optima at 6.3, 7.4 and 8.5, these results were obtained at pH 8.5. Work is in progress to ascertain if the enzymes more sensitive to DNP have greater activity at lower pH values.

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[1] F. E. HUNTER, in W. D. MCELROY AND H. B. GLASS, *Phosphorus Metabolism*, Vol. I, Johns Hopkins Press, Baltimore, 1951, p. 297.
[2] H. A. LARDY AND H. WELLMAN, *J. Biol. Chem.*, 201 (1953) 357.
[3] C. COOPER AND A. L. LEHNINGER, *J. Biol. Ghem.*, 224 (1957) 561.
[4] R. PENNIALL, *Analytical Biochem.*, in the press.
[5] D. K. MYERS AND E. C. SLATER, *Biochem. J.*, 67 (1957) 558.

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Nitrite reductase activity of *Pseudomonas* cytochrome oxidase

As is well known, *Pseudomonas aeruginosa* can be cultivated under anaerobic as well as under aerobic conditions, if nitrate is present. Thus the bacterium is able to use nitrate as an electron acceptor under anaerobic conditions, as well as oxygen under aerobic conditions; this is called nitrate respiration. From the bacterium, P-cytochrome *c*-551, P-cytochrome-554, P-cytochrome oxidase and P-blue protein have been extracted and purified, and P-cytochrome *c*-551 and P-blue protein obtained crystalline[1–5]. Another cytochrome, P-cytochrome-(560), which has its α-absorption band at 560 mμ, has also been found in the bacterium[1,6]. These respiratory components have been shown to function in nitrate respiration as well as in oxygen respiration of the organism[6]. P-cytochrome oxidase, which is almost pure ultracentrifugally and 70 % pure electrophoretically, contains two haem moieties, haem a_2 and a *c*-type haem[7,8], and oxidizes reduced P-cytochrome *c*-551 and P-blue protein, but not reduced P-cytochrome-554 or mammalian cytochrome *c*[2,7]. Like animal cytochrome oxidase, the enzyme also oxidizes L-ascorbate and hydroquinone. The oxygen consumption in the above reactions is inhibited strongly by a small amount of nitrite, but not by nitrate. This suggests either that nitrite is an inhibitor of P-cytochrome oxidase or that an electron destined for oxygen in the absence of nitrite is captured by nitrite. When nitrite and the oxidase were present together, reduced P-cytochrome *c*-551 could also be oxidized rapidly under anaerobic conditons, as shown in Fig. 1, and a part of nitrite present in the reaction mixture disappeared. Neither nitrate nor hydroxylamine could replace nitrite. This indicates that reduced P-cytochrome *c*-551 donates an electron to nitrite in the presence of P-cytochrome oxidase. This reaction was about 80 % inhibited by 10^{-3} *M* cyanide, which had been found to give complete inhibition of the oxidase activity under aerobic conditions. Heat treatment (70° for 5 min) of P-cytochrome

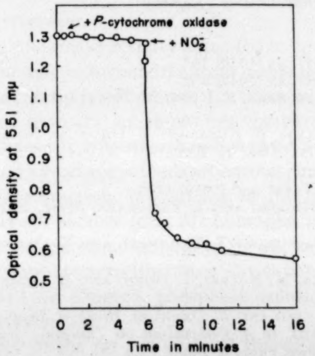

Fig. 1: Oxidation of reduced P-cytochrome *c*-551 by nitrite in the presence of P-cytochrome oxidase under anaerobic conditions. Reactions were carried out in a Thunberg tube modified for spectrophotometry. The tube has two side chambers. After the tube had been evacuated and filled with N_2, reduced P-cytochrome *c*-551 and P-cytochrome oxidase were mixed. The absorbancy at 551 mμ decreased very slightly. After 6 min, nitrite was added to the reaction mixture, bringing about a rapid oxidation of reduced P-cytochrome *c*-551. When P-cytochrome oxidase was absent, nitrite could not oxidize reduced P-cytochrome *c*-551, under the experimental conditions: pH 7.0, 28°.

oxidase diminished the velocity of the reaction to a tenth of the original. Hydroquinone could also act as electron donor for the reduction of nitrite. Nitrite was also reduced with yeast lactate dehydrogenase, lactate and P-cytochrome *c*-551 as an electron-donor system. If phenazine methosulphate was used instead of P-cytochrome *c*-551, nitrite reduction was much accelerated. However, in this case, cyanide did not inhibit. Pyocyanine, which is a pigment produced by the bacterium, could replace phenazine methosulphate, which may be a clue towards elucidating the physiological function of pyocyanine in the organism. None of the following haemoproteins could replace P-cytochrome oxidase in this reaction: P-cytochrome *c*-551, mammalian cytochrome *c*, cytochrome ***c*** modified by treatment with trichloroacetic acid[9], cytochrome ***a***, a mixture of cytochrome ***a*** and cytochrome *c* which shows cytochrome oxidase activity[10], and catalase. With the hand spectroscope, P-cytochrome oxidase reduced with ascorbate could be seen to be oxidized immediately under anaerobic conditions when nitrite was added.

VERHOEVEN AND TAKEDA[11] observed that a reduced "cytochrome *c*"-type pigment in a sonicate of *Pseudomonas aeruginosa* was oxidized by addition of nitrite. The pigment may be identical with P-cytochrome *c*-551. Our results with highly purified cytochrome components have extended these findings and have shown that P-cytochrome oxidase functions as nitrite reductase. This is in good accordance with the fact that P-cytochrome oxidase can be extracted in greater amounts from cells cultivated under anaerobic than from those grown under aerobic conditions.

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[1] T. HORIO, *J. Biochem. (Tokyo)*, 45 (1958) 195.
[2] T. HORIO, *J. Biochem. (Tokyo)*, 45 (1958) 267.
[3] T. HORIO, T. HIGASHI, H. MATSUBARA, K. KUSAI, M. NAKAI AND K. OKUNUKI, *Biochim. Biophys. Acta*, 29 (1958) 297.
[4] T. HORIO, T. HIGASHI, M. NAKAI, K. KUSAI AND K. OKUNUKI, *Nature*, 182 (1958) 1307.
[5] T. HORIO, T. HIGASHI, M. SASAGAWA, K. KUSAI, M. NAKAI AND K. OKUNUKI, *Biochem. J.*, 77 (1960) 194.
[6] T. YAMANAKA, *J. Biochem. (Tokyo)*, 46 (1959) 1289.
[7] K. OKUNUKI, T. HORIO, T. HIGASHI AND T. YAMANAKA, *Symp. Enzyme Chem.*, (in Japanese), in the press.
[8] T. HORIO, T. HIGASHI, H. MATSUBARA, T. YAMANAKA AND K. OKUNUKI, *J. Biol. Chem.*, in the press.
[9] T. YAMANAKA, H. MIZUSHIMA, M. NOZAKI, T. HORIO AND K. OKUNUKI, *J. Biochem. (Tokyo)*, 46 (1959) 121.
[10] I. SEKUZU, S. TAKEMORI, Y. ORII AND K. OKUNUKI, *Biochim. Biophys. Acta*, 37 (1960) 64.
[11] W. VERHOEVEN AND Y. TAKEDA, in *A Symposium on Inorganic Nitrogen Metabolism*, Johns Hopkins Univ. Press, Baltimore, 1956, p. 159.

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Activation of long-chain fatty acids by rat-gut mucosa

The esterification of long-chain fatty acids has been shown to be an important phase of intestinal fat absorption. Demonstration that the incorporation of free fatty acids into glycerides by homogenates of human- and rat-gut mucosa[1] is dependent on CoA, ATP and Mg^{++} suggested that the initial step in the process might be activation of the fatty acids to acyl-CoA thioesters. Such a step occurs in the liver in formation of phosphatidic acid intermediates[2] from which triglycerides and phospholipids are thought to be derived[3].

Although it is possible that to some extent monoglycerides, at least, may be absorbed intact without hydrolysis[4,5], current evidence indicates that the major pathway of intestinal fat absorption involves the mucosal transport of free fatty acids, with subsequent esterification into chylomicron triglyceride[6]. Therefore, the presence in the gut of a system capable of synthesizing CoA derivatives of long-chain fatty acids was not unexpected.

Studies on homogenates of rat proximal jejunal mucosa and subcellular particles obtained therefrom have shown a very active long-chain fatty acid thiokinase requiring CoA, ATP and Mg^{++}, similar to that found in liver[2] and adipose tissue[7] (Reaction I).

$$\text{R–COOH} + \text{ATP} + \text{CoA} \xrightleftharpoons{Mg^{++}} \text{R–CO}{\sim}\text{CoA} + \text{AMP} + \text{PP} + H_2O \qquad \text{(I)}$$

$$\text{R–CO}{\sim}\text{CoA} + NH_2OH \longrightarrow \text{R–CONHOH} + \text{CoA} \qquad \text{(II)}$$

Assay of this thiokinase was carried out using NH_2OH as a trapping agent for the acyl-CoA produced (Reaction II) and spectrophotometric estimation of the ferric-hydroxamic acid complexes by absorption at 520 mμ ($\varepsilon_M = 1.28 \cdot 10^3$). Alternatively, using [1-^{14}C]palmitate as a substrate, the resultant labeled palmithydroxamic acid was separated on anion-exchange resin paper (IRA-400) and counted directly in a liquid scintillation spectrometer.

Maximal palmitate thiokinase activity appeared to be localized in the microsomal fraction (Table I), although mitochondria from neutral-isotonic-KCl homogenates also showed considerable activity. The latter particles were rather heavily contaminated with microsomes, however, as shown by comparative alkaline phosphatase activities[8]. The microsomes, on the other hand, seemed almost free of mitochondria by phase microscopy and cytochrome oxidase[9] estimations. Virtually no activity was found in the supernatant fraction, in contrast to the findings of KORNBERG AND PRICER in guinea-pig liver[2]. Sucrose (0.25 *M*) homogenates of gut mucosa, although providing more cleanly separated subcellular particles, were considerably less active and the diminished particulate activities were not enhanced by washing and resuspension in isotonic KCl or Ringer's phosphate solution. Additional media and conditions are being investigated.

The specific activity of the rat-gut mucosal thiokinase was several-fold greater in the duodenum and proximal jejunum than in the distal intestine and was not impaired by 48 h of fasting. Although the specific activity of the microsomal preparations varied somewhat, it was in general of the same order of magnitude as reported for for guinea-pig liver microsomes[2], namely, 0.2 to 1.0 μmoles/h/mg protein. Homogenates

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Abbreviations: CoA, coenzyme A; ATP, adenosine triphosphate; AMP, adenosine monophosphate; PP, pyrophosphate; GSH, glutathione; Tris, tris(hydroxymethyl)aminomethane.

TABLE I

DISTRIBUTION OF A FATTY ACID THIOKINASE ACTIVITY IN RAT-GUT MUCOSA

The reaction system contained 0.5 ml suspended enzyme preparation (2–4 mg protein/ml), 2.0 µmoles palmitate (or [1-^{14}C] palmitate, 140,000 counts/min/µmole) in 5% bovine albumin, 20 µmoles ATP, 20 µmoles $MgCl_2$, 50 µmoles KF, 1 mg CoA (approximately 70% pure), 20 µmoles GSH, 1 mmole NH_2OH and 125 µmoles Tris–HCl, pH 7.4, in a total volume of 2.5 ml. Incubation was at 37° in air, for 30 min, and was stopped by chilling in ice and adding 0.2 ml 21% $HClO_4$. Lipids were extracted with $CHCl_3$–CH_3OH (2:1), separated from water-soluble material[10], and isolated for assay by evaporation of solvent with air or N_2 at 40°.

Fraction	Protein		Palmitate thiokinase
	mg/g wet mucosa	*% of total*	*µmoles hydroxamate/h/mg protein*
Whole homogenate, 5% (w/v)	44	100	0.082
Nuclei			
10 min at 2,000 × *g*, twice	16	36	0.075
Mitochondria			
20 min at 7,500 × *g*, twice	4	9	0.150
Microsomes			
30 min at 105,000 × *g*,	5	11	0.287
Supernatant	18	41	0.001

* Homogenates and fractions were prepared at 0–4°, washed and resuspended in 0.154 *M* KCl (pH 7.4, using 0.1 *N* KOH) or 0.25 *M* sucrose.

** Protein was measured by the biuret or Folin–Ciocalteau method[11].

and particles were capable of activating fatty acids other than palmitic, with comparable effectiveness, including stearic, oleic, myristic, and to a lesser extent, lauric and shorter-chain acids. A rather broad pH optimum between 6.8–7.6 was found. Taurocholate ($5 \cdot 10^{-3}$ *M*) almost completely abolished microsomal activity.

The demonstration in rat-gut mucosa of a long-chain fatty acid thiokinase, with greatest activity in the microsomal fraction may be relevant to the electron-microscopic observations of PALAY AND KARLIN[12] that pinocytosis is involved in intestinal fat absorption. Since in this process transported lipid is surrounded by endoplasmic reticulum (microsomes), fatty acid activation and possibly further reactions in glyceride synthesis may occur while the lipid is within the pinocytotic vesicle.

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[1] A. M. DAWSON AND K. J. ISSELBACHER, *J. Clin. Invest.*, 39 (1960) 150.
[2] A. KORNBERG AND W. E. PRICER JR., *J. Biol. Chem.*, 204 (1953) 329.
[3] S. WEISS AND E. P. KENNEDY, *J. Am. Chem. Soc.*, 78 (1956) 3550.
[4] R. REISER AND M. C. WILLIAMS, *J. Biol. Chem.*, 202 (1953) 815.
[5] V. P. SKIPSKI, M. G. MOREHOUSE AND H. J. DEUEL JR., *Arch. Biochem. Biophys.*, 8[illegible] (1959) 93.
[6] H. C. TIDWELL AND J. M. JOHNSTON, *Arch. Biochem. Biophys.*, 89 (1960) 79.
[7] D. STEINBERG, M. VAUGHAN, S. MARGOLIS AND A. KARMEN, *Fed. Proc.*, 19 (1960) 227.
[8] R. K. MORTON, *Biochem. J.*, 57 (1954) 595.
[9] F. APPELMANS R. WATTIAUX AND C. DE DUVE, *Biochem. J.*, 59 (1959) 438.
[10] J. FOLCH, M. LEES AND G. H. SLOANE STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
[11] E. LAYNE, in *Methods in Enzymology*, Vol. III, Academic Press Inc., New York, 1957, p. 447.
[12] S. PALAY AND L. J. KARLIN, *J. Biophys. Biochem. Cytol.*, 5 (1959) 373.

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SELF-DIFFUSION OF WATER IN TOBACCO MOSAIC VIRUS SOLUTIONS

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SUMMARY

Self-diffusion coefficients for water in solutions of tobacco mosaic virus have been measured near the critical concentration of the solutions. The diffusion coefficients of the water in the solutions is very close to the diffusion coefficient of water in pure water. It is concluded from this data that no more than 3 % of the water in a 4 % tobacco mosaic virus solution is in a bound ice-like state. This statement is in disagreement with JARDETSKY and JARDETSKY who conclude from nuclear magnetic resonance measurements that as much as 20 % of the water in a 1.9 % tobacco mosaic virus solution exists in an ice-like structure.

The self-diffusion coefficients of protons in aqueous solutions of T.M.V. near the critical concentration have been measured using a nuclear magnetic resonance spin-echo technique. The spin-echo apparatus, which uses high intensity pulsed radio frequency resonant radiation, as opposed to the usual steady state N.M.R., which uses very small and steady intensity resonant radiation, is described in ref. 1. The spin-echo technique is described in ref. 2, 3 and details of the technique used herein for the measurement of self-diffusion coefficients are discussed in ref. 4.

The sample of 80–85 % monodisperse T.M.V. was obtained through the courtesy of Dr. W. M. STANLEY at the University of California. The initial concentration of the sample was 26.9 mg T.M.V./ml of solution containing traces of phosphate buffer at pH 7. At this concentration, no phase separation appeared during six months at 4°. A part of the initial sample was diluted with distilled water to a concentration of 10.8 mg T.M.V./ml of solution. A second part of the sample was concentrated by slow evaporation of water on a house vacuum line to a concentration of about 40.4 mg T.M.V./ml of solution. After several weeks the 40.4 g/ml solution separated with about 0.2 volume fraction of dense phase. After completion of the measurements at 40.4 mg/ml, this same sample was concentrated further until it was all dense phase.

Proton signal intensity measurements relative to a water sample were made and indicated that no appreciable fraction of the protons in the sample resided in an ice-like structure with relaxation times so short that they were not observed.

The data in Fig. 1 shows that the self-diffusion coefficients of protons in all the T.M.V. solutions are within 2–3 % of the self-diffusion coefficients of protons in pure

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Abbreviation: T.M.V. tobacco mosaic virus.

water. In both water and T.M.V. the proton diffusion coefficients almost surely represent the diffusion coefficient of water molecules[5].

The magnitudes of the differences in proton diffusion coefficients in pure water and T.M.V. solutions are about equal to the estimated magnitude of the experimental error. Nevertheless, it is worth noting that diffusion coefficients of water in T.M.V. solutions are consistently lower than the diffusion coefficient of water in pure water. The activation energy of the diffusion coefficient in pure water agrees well with that obtained by SIMPSON AND CARR[6]. Fig. 2 shows the temperature-dependence of the proton diffusion coefficient for pure water and for the most concentrated T.M.V. solution.

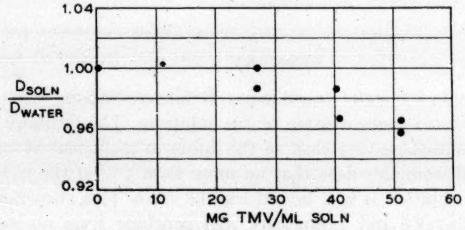

Fig. 1. Diffusion coefficients of T.M.V. solutions relative to water at room temperature *vs.* concentration of the T.M.V. solution.

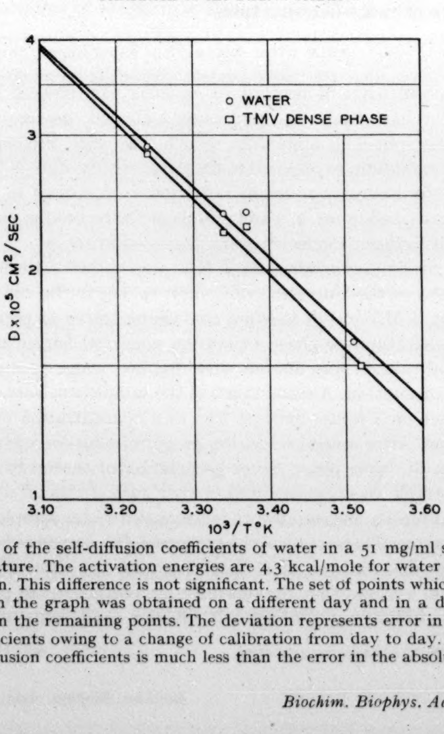

Fig. 2. Logarithm of the self-diffusion coefficients of water in a 51 mg/ml solution of T.M.V. *vs.* reciprocal temperature. The activation energies are 4.3 kcal/mole for water and 4.5 kcal/mole for the T.M.V. solution. This difference is not significant. The set of points which deviates most from the lines drawn on the graph was obtained on a different day and in a different region of the magnetic field than the remaining points. The deviation represents error in the absolute value of the diffusion coefficients owing to a change of calibration from day to day. The relative error for the diffusion coefficients is much less than the error in the absolute values.

It has been suggested that long range order of solutions of T.M.V. in water arises from the interaction of hydration shells of the virus particles[7,8]. This attitude is in contrast with the theory of organization of rod-shaped particles advanced by ONSAGER[9] and elaborated for T.M.V. solutions by OSTER[10]. Indeed, JARDETZKY AND JARDETZKY[11] suggested that their N.M.R. results are consistent with an interpretation in which about 20% of all the water in the solution is organized into an ice-like structure around the T.M.V. particles.

If water tends to associate into an ice-like structure, or even a more weakly associated complex, an average molecule is immobilized* for a certain fraction, f, of the time of observation in the experiment. On the basis of this rather simple model one can write the self-diffusion coefficient, D, of the water in the T.M.V. solution as

$$D = D_0 \{ 1 - [1 - (\nu_b/\nu_0)f] \}$$

where D_0 is the self-diffusion coefficient of water in pure water, ν_b is the diffusion jump frequency of the water of hydration (bound water) and ν_0 is the jump frequency of water in pure water. If the binding is at all appreciable ν_b/ν_0 is much less than one and $D/D_0 \cong 1\text{-}f$. Since the system is at equilibrium, the time average f is also the average fraction of bound water molecules at any given time. The data and this result give a lower bound for $1\text{-}f$ and hence an upper bound for f of about 3% bound water. Taking the molecular weight of the T.M.V. to be about 40 million, one finds that this corresponds to $1.6 \cdot 10^6$ water molecules per virus molecule in a 4% T.M.V. solution. This number of water molecules per virus molecule does not exclude what might be considered the normal hydration of the roughly 50,000 ionic groups per T.M.V. molecule**.

The spin-lattice relaxation time T_1 and the spin-spin relaxation time T_2 were also measured and are approximately 0.75 and 0.20 sec respectively for the sample containing 26.9 mg T.M.V./ml. We consider it probable that these relaxation times are to a large degree determined by paramagnetic effects and, as such, are difficult to relate to the problem discussed here.

REFERENCES

[1] J. SCHWARTZ, *Rev. Sci. Instr.*, 28 (1957) 780.
[2] E. L. HAHN, *Phys. Today*, 6 (1953) 4.
[3] H. Y. CARR AND E. M. PURCELL, *Phys. Rev.*, 94 (1954) 630.
[4] D. C. DOUGLASS AND D. W. MCCALL, *J. Phys. Chem.*, 62 (1958) 1102.
[5] D. C. DOUGLASS AND D. W. MCCALL, *J. Chem. Phys.*, 31 (1959) 569.
[6] O. H. SIMPSON AND H. Y. CARR, *Phys. Rev.*, 111 (1958) 1201.
[7] J. D. BERNAL AND I. FANKUCHEN, *J. Gen. Physiol.*, 25 (1941) 111.
[8] G. OSTER, *J. Cellular Comp. Physiol.*, 49 Supplement 1 (1957) 124.
[9] L. ONSAGER, *Ann. N.Y. Acad. Sci.*, 51 (1949) 627.
[10] G. OSTER, *J. Gen. Physiol.*, 33 (1950) 445.
[11] C. D. JARDETZKY AND O. JARDETZKY, *Biochim. Biophys. Acta*, 26 (1957) 668.

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* The random translational degrees of freedom of the center of mass of the water molecule are converted into multiply-periodic vibrational degrees of freedom.

** We are indebted to Professor I. M. Klotz for pointing this fact out to us and thereby correcting an erroneous statement in our manuscript.

THE MECHANISM OF REACTIVATION OF ENZYME SYSTEMS IN MITOCHONDRIAL PREPARATIONS TREATED WITH ORGANIC SOLVENTS

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SUMMARY

The mechanism of inactivation of respiratory enzyme systems in mitochondrial preparations on treatment with organic solvents has been investigated. It was found that the inactivation of the enzyme systems could be attributed mainly to the presence of residual solvent which acted as a physical inhibitor. This inhibition could be reversed by any one of a number of different treatments which removed the solvent. It was shown that the specific reactivations claimed by other workers for substances such as tocopherol can be explained on the basis of non-specific reversal of this inhibition. Suitable techniques for studying preparations in which lipid has been removed by extraction, but without the complicating effect of residual solvent, will be described. The results will be discussed in relation to previous work.

INTRODUCTION

It was shown by NASON AND LEHMAN[1,2] that extraction of muscle mitochondrial preparations with isooctane resulted in an almost complete loss of the cytochrome *c* reductase activities. They could, however, be restored to their original levels by treating the preparations with α-tocopherol as a suspension in bovine serum albumin. It was suggested, therefore, that tocopherol was either an essential component of the respiratory chain or that it could replace some lipid cofactor which was assumed to act between cytochromes *b* and *c*. It was subsequently found by NASON and his group[3] and by others[4] that a number of substances unrelated to tocopherol would reactivate extracted enzyme preparations. The original hypothesis was not abandoned however, and the results were explained on the basis of a specific reactivation by tocopherol[5]. DEUL, SLATER AND VELDSTRA[6] repeated the work of NASON's group and concluded that the reactivation by tocopherol was best explained as a non-specific physical reversal of an inhibition caused by isooctane which was adsorbed on the surface of the enzyme particle. Experimental support for this idea came from the work of REDFEARN AND PUMPHREY[7] who showed that the non-ionic surface-active agent, Tween 80, was a more efficient reactivator than *d*-α-tocopherol in the succinic–cytochrome *c* reductase system of a pig heart-muscle preparation. It seemed, therefore,

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Abbreviations: DPNH, reduced diphosphopyridine nucleotide; Tris, tris(hydroxymethyl)-aminomethane.

that the principal mechanism of reactivation was the displacement or dispersal of the residual organic solvent which was adsorbed on or associated with the enzyme particles so as to prevent access of the reactants, or to inhibit electron transfer at some point in the respiratory chain, or both. This type of reactivation mechanism would also explain the reactivating effect of a large number of substances, all possessing long isoprenoid chains, studied by Weber, Gloor and Wiss[8]. Further evidence in favour of this mechanism came from Pollard and Bieri[9] who found that mechanical treatment, such as high-speed centrifugation or freeze-drying, would effectively remove residual solvent and produce a reactivation.

At the present time, a great deal of interest is being focussed on the possible roles of lipids as structural and functional components of the electron transport system, and a suitable extraction–reactivation technique would seem to be a useful tool for these studies. It appears, however, that the predominating factor in the inactivation of enzyme systems in preparations treated with organic solvents is a physical inhibition caused by the residual solvent. Previous claims for the participation of certain lipid co-factors in the respiratory chain can now be explained on the basis of non-specific reactivations by the reversal of the inhibitory effects of the solvent and not to the replacement of specific lipids removed by the extraction. If the extraction–reactivation technique is to be used as a valid test system for studying the structural and functional lipids of the respiratory chain then the inhibitory solvent must first be removed.

In the present paper the results of a study of the mechanisms of extraction and reactivation of mitochondrial enzyme systems will be presented and discussed.

MATERIALS AND METHODS

Solvents: A.R. isooctane and light petroleum (b.p. 40–60°) were freed from benzene by passing them through a column of silica gel (200 mesh) and then distilling.

Chemicals: Cytochrome *c* was prepared from ox heart[10] and dialysed against water. Ubiquinone was obtained from pig heart[11]. *d*-α-tocopherol was prepared from the acetate by saponification. DPNH was purchased from Boehringer und Soehne, Mannheim, Tween 80 from Honeywell and Stein, London, and bovine serum albumin from Armour and Co., Eastbourne.

Tocopherol and ubiquinone suspensions: These were prepared by dissolving ubiquinone (3 mg) or *d*-α-tocopherol (1.5 mg) in ethanol (1.2 ml). 4.8 ml of an aqueous solution of bovine serum albumin (0.2 %, w/w) were then added and the mixture shaken vigorously by hand. The final concentrations were $5.8 \cdot 10^{-4}$ *M*.

Heart-muscle preparations: Two types, I and II, were used. These were prepared by procedures described by Morrison, Crawford and Stotz[12] and Pumphrey and Redfearn[13], respectively.

Final protein concentrations[14] in both types of preparation varied between 10 and 40 mg/ml.

Extraction procedure: Unless otherwise stated, the method used was as follows:

The enzyme preparation was shaken vigorously by hand with an equal volume of isooctane, or other solvent, for 1 min at room temperature. The organic solvent and aqueous layers were then separated by giving the tube a short spin in a clinical centrifuge. The upper organic solvent layer was then removed.

Measurement of enzyme activities

Succinic-cytochrome c *reductase* was measured spectrophotometrically at 550 mμ at room temperature (17–22°) as already described[7], and the reaction started by adding the enzyme.

DPNH-cytochrome c *reductase* was measured in the same way as the succinic cytochrome *c* reductase except that the succinate was replaced by DPNH ($1.1 \cdot 10^{-4} M$).

Succinic oxidase was measured manometrically at 37° with a reaction mixture as previously described[15]. The taps were closed 5 min after placing the manometer in the bath, and the succinic oxidase activity was calculated from the oxygen uptake in the 5 to 15-min period after closing the taps.

In order to study the effect of solvent treatment on the succinic oxidase activity it was necessary to devise a method for removing the residual solvent before carrying out the assay. Interference due to evolution of vapour from residual solvent would thus be avoided. The method used was first to blow a stream of nitrogen over the extracted preparation for a few minutes at room temperature. The preparation, after suitable dilution, was then placed in the Warburg flask with a small concentration of succinate (0.004 *M*) and with all the other additions. The remainder of the succinate addition was placed in the side-arm. The flask was then shaken in the water-bath at 37° with the manometer tap open until all the solvent vapour had been evolved. The succinate was then tipped into the reaction mixture and the rate of oxygen uptake measured. The unextracted controls were treated in the same way.

DPNH oxidase was measured spectrophotometrically at 340 mμ at room temperature (17–22°) using the same reaction mixture as used for DPNH–cytochrome *c* reductase except that the cyanide addition was omitted.

Cytochrome oxidase was measured manometrically at 37° with the following reaction mixture: KH_2PO_4–Na_2HPO_4 or Tris–HCl buffer, pH 7.4, 0.07 *M*; *p*-phenylene diamine, 0.04 *M*; cytochrome *c*, $7 \cdot 10^{-5} M$; heart-muscle preparation, 0.25–1.0 mg protein. The final reaction volume of the flask contents was 1.4 ml. The gas phase was air. The cytochrome oxidase activity was calculated from the oxygen uptake in the 5–15 min period.

RESULTS

General observations

It will be shown that treatment of the preparations with organic solvents can affect enzyme activities in several ways, quite apart from the removal of lipid. In this paper, the term "extraction" will be used to denote the shaking of the preparation with organic solvent regardless of the quantity of lipid removed.

The effects of extraction on enzyme activities depended on a number of factors such as the type of preparation, its age and method of storage, the nature of the solvent and the amount of solvent retained in the preparation after the extraction procedure. Most of these factors will be dealt with in the results of experiments on the different enzyme systems. However, some general observations on the extraction procedure might be described here since they give a clue to an understanding of the mechanism of inactivation by solvents.

In the extraction procedure, the vigorous shaking of the preparation with the solvent usually resulted in emulsion formation. After centrifugation, the aqueous and solvent layers had separated and most of the enzyme particles had floated to the top of the aqueous layer instead of sedimenting or remaining dispersed. It appeared that the solvent had become adsorbed on the surface or had dissolved in the lipid of the particle and so lowered its density. If the upper solvent layer was removed and the particles redispersed in the aqueous layer it was found that there was an evolution of solvent vapour from the suspension. When all the residual solvent had been evolved centrifugation of the suspension caused a sedimentation of the particles.

Evidence that the solvent itself is the principal cause of inactivation was provided by shaking the heart-muscle preparation with a small volume (10–20 % v/v) of the solvent. Measurement of succinic cytochrome *c* reductase activity of the mixture then showed that a considerable inactivation had occurred.

Experiments were performed on two types of preparation, I and II. The essential differences between them were that type I contained ammonium sulphate and it was stored at —20° for periods up to several weeks while type II did not contain ammonium sulphate and was stored at 3–5° for 6–7 days. In a few experiments, ammonium sulphate was added to a type II preparation which was then kept at —20°. This treatment gave a preparation which appeared similar to that of type I. The long period of storage of this type at —20° appeared to make it more sensitive to treatment with organic solvents; *i.e.* greater enzyme inactivations were obtained after extraction and removal of residual solvent than those obtained with the type II preparations.

Effects of solvent treatment on cytochrome c *reductase activities*

Succinic- and DPNH-cytochrome *c* reductase activities were measured in both types of preparation. The loss in activities of the heart-muscle preparation after one extraction increased after storage at —20°. NASON AND LEHMAN[2] had also found that enzyme inactivation by extraction was facilitated by storage of their preparation. Fig. 1 shows the succinic- and DPNH-cytochrome *c* reductase activities of a preparation before and after a single extraction with isooctane as a function of the age of the preparation. The isooctane extract had an absorption maximum at about 270 mμ and as a rough index of the amount of lipid extracted, the extinction at 270 mμ was measured.

The reactivating effect of *d*-α-tocopherol[1,2,16] on the succinic- and DPNH-cytochrome *c* reductase activities was confirmed. It was found also that ubiquinone (coenzyme Q) would reactivate, a result which is in agreement with work of another group[8]. It seemed that these substances could be acting by virtue of their surface-active properties in removing residual solvent which was inhibiting the enzyme systems. The effect of adding the non-ionic surface-active agent, Tween 80 was therefore tried and it was found that it gave 100 % reactivation in the succinic-cytochrome *c* reductase activity of an isooctane-extracted preparation (Fig. 2). Tween 80 in the concentration used had little or no effect on succinic-cytochrome *c* reductase in the unextracted preparation.

In another experiment a preparation of type I was aged for 2 weeks at —20°, and then it was successively extracted with isooctane up to 10 times. The reactivating effect of Tween 80, ubiquinone and *d*-α-tocopherol were tested after each extraction (Table I). Up to three extractions seemed to have little effect on the maximal restored activity, although as much as 34 % (by weight) of the total lipid had been removed. After three extractions the restored activities fell rapidly until with ten extractions, when 49 % of the lipid had been removed, the maximal restored activity had fallen to 42 % of the original activity.

In experiments on DPNH-cytochrome *c* reductase, it was found that *d*-α-tocopherol and ubiquinone would reactivate after extraction, but Tween 80 would not. In fact, Tween 80 destroyed almost all activity in the unextracted preparation. This is in keeping with the greater sensitivity of the DPNH–cytochrome *c* reductase

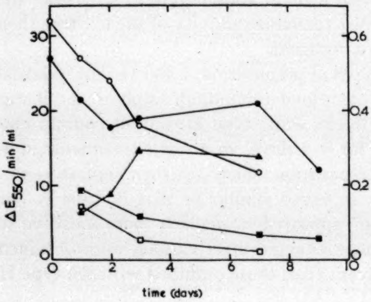

Fig. 1. Effect of one extraction with isooctane on succinic- and DPNH–cytochrome *c* reductase activities of a type I heart-muscle preparation as a function of the age of the preparation. ○—○, DPNH–cytochrome *c* reductase before extraction; ●—●, succinic–cytochrome *c* reductase before extraction; □—□, DPNH–cytochrome *c* reductase after extraction; ■—■, succinic–cytochrome *c* reductase after extraction; ▲—▲, extinction at 270 mμ of isooctane extract per ml undiluted preparation. Enzyme activities are expressed as rate of change of extinction at 550 mμ/ml undiluted preparation. Assay procedures as described in the text.

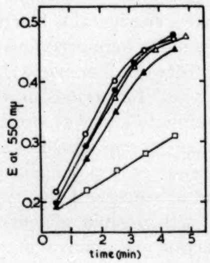

Fig. 2. Reactivation of succinic–cytochrome *c* reductase by *d*-α-tocopherol, ubiquinone and Tween 80. ○—○, unextracted; □—□, extracted once with isooctane; △—△, extracted plus *d*-α-tocopherol, 3.8·10⁻⁵ *M*; ▲—▲, extracted plus ubiquinone, 3.8·10⁻⁵ *M*; ●—●, extracted plus Tween 80, 1.33 μg/ml. Type II preparation with added ammonium sulphate (8.8% w/v) aged at —20° for 7 days. *d*-α-tocopherol and ubiquinone added as suspensions in 0.2% bovine serum albumin. Assay procedure as described in text.

TABLE I

EFFECT OF ISOOCTANE EXTRACTION ON THE SUCCINIC-CYTOCHROME *c* REDUCTASE OF A PIG HEART-MUSCLE PREPARATION

Extraction and assay as described in text. Type I preparation (age: 2 weeks) used. Final concentrations of additions: Tween 80, 1.33 mg/ml; *d*-α-tocopherol, $3.8 \cdot 10^{-5}$ *M*; ubiquinone, $3.8 \cdot 10^{-5}$ *M*. Suspensions of *d*-α-tocopherol and ubiquinone in 0.2 % bovine serum albumin were used.

Number of extractions	*Additions:*	*Relative activity*			
		None	*Tween 80*	*d-α-tocopherol*	*Ubiquinone*
0		100	100	100	100
1		17	108	78	84
2		11	103	70	81
3		11	100	58	80
4		9	89	52	77
7		7	60	22	51
10		2	42	13	26

system to surface-active agents and organic solvents already noted by other workers[6,17].

Effect of solvent-treatment on succinic oxidase activity

The manometric determination of succinic oxidase after treatment of the preparation with organic solvents is complicated by the fact that there is an evolution of vapour from the residual solvent during the course of the assay. After a time, when all the solvent has been driven off, the manometer indicates an uptake of oxygen. These effects are illustrated in Fig. 3.

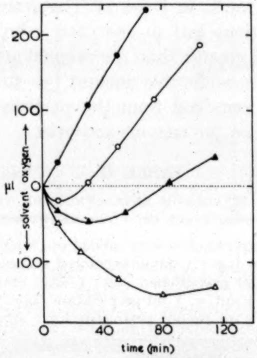

Fig. 3. Succinic oxidase activity after 1, 4 and 9 extractions with isooctane. ●—●, unextracted; ○—○, 1 extraction; ▲—▲, 4 extractions; △—△, 9 extractions. Type II preparation with added ammonium sulphate (8.8 % w/v) aged at —20° for 7 days. Assay procedure as described in text.

The effects of *d*-α-tocopherol, ubiquinone and Tween 80 additions on the succinic oxidase activities of extracted preparations were studied. Tween 80 appeared to accelerate the evolution of the residual solvent and consequently the oxygen uptake began earlier. Ubiquinone and tocopherol as suspensions in bovine serum albumin did not give this effect but often tended to eliminate part of the "lag-period" due to solvent evolution, possibly by preferentially adsorbing the solvent; this would reverse the inhibition and perhaps also tend to prevent the solvent from evaporating. None of these substances had any effect on the final maximum activity after extractions.

The amount of residual solvent in a preparation depends upon a number of factors, such as the number of extractions and the dilution after extraction. If the amount of solvent is small and the succinic oxidase activity high, then there will be apparent uptake of oxygen but this will be lower than the true activity. When all the solvent has been driven off, the apparent oxygen uptake will attain its maximal true value. A plot of the "oxygen uptake" against time will thus give a curve with an apparent lag period similar to those published by DEUL, SLATER AND VELDSTRA[6]. The interfering effect of the evolution of residual solvent vapour can, however, be eliminated by using the modified procedure described above in which the residual solvent is removed before the assay. Using this procedure the effect of the number of extractions and the addition of cytochrome *c*, tocopherol and ubiquinone on the succinic oxidase activity of the preparations after solvent treatment was studied.

Number of extractions: The effects of an increasing number of extractions of a type II preparation which had been aged at —20° for 1 week have already been shown (Fig. 3). It was found that there was a progressive loss of activity with an increasing number of extractions with isooctane. Subsequent experiments employing the modified technique for removing residual solvent were done with unfrozen type II preparations using the more volatile 40–60° light petroleum as the solvent (Table II). It can be seen that after one or two extractions there was an increase in the succinic oxidase activity (> 100 % in Expt. 1). The activities then fell slowly with increasing number of extractions but in one case, even after 40 extractions, the activity was more than 100 % greater than the original activity. Preliminary experiments have indicated that a considerable amount (30–50 %) of the ubiquinone and possibly other lipids can be removed from the particles apparently without this having any appreciable effect on the enzyme activities.

TABLE II

EFFECT OF NUMBER OF LIGHT PETROLEUM EXTRACTIONS ON THE SUCCINIC OXIDASE AND CYTOCHROME OXIDASE ACTIVITIES OF A PIG HEART-MUSCLE PREPARATION

Assays as described in text. The experiments were carried out with three different type II preparations. Expt. 1: 2 ml preparation (age: 3 days) extracted successively with 4 ml 40–60° light petroleum for 1 min. Expt. 2: 1 ml preparation (age: 1 day) extracted successively with 1 ml 40–60° light petroleum for 1 min. Expt. 3: 1 ml preparation (age: 7 days) extracted successively with 5 ml 40–60° petroleum for 1 min.

Number of extractions	*Succinic oxidase QO_2 (µl O_2/h/mg protein)*			*Cytochrome oxidase QO_2*	
Expt. No.	*1*	*2*	*3*	*1*	*3*
0	259	216	320	810	1240
1	567	293	—	886	—
2	373	297	—	842	—
3	—	288	—	—	—
4	243	—	—	902	—
5		225	—		
7		220	—		—
10		135	—		—
20			960		1616
40			770		1880

Cytochrome c *addition*: In all these succinic oxidase assays, cytochrome *c* was added. This was necessary because extraction of these preparations with organic solvents appeared to increase their dependency on external cytochrome *c* for attainment of maximal activity. If the succinic oxidase activity was measured without added cytochrome *c*, after extraction there was an apparent loss in activity which could be restored by adding cytochrome *c*. Fig. 4 shows the effect of cytochrome *c* addition on a fresh heart-muscle preparation before and after one extraction with light petroleum.

d-*α-tocopherol addition*: The effects of the addition of *d-α*-tocopherol either as a solution (ethanol) or a suspension (bovine serum albumin) were studied. The results of one experiment are shown in Fig. 5. A Tris-buffered preparation of type II was extracted four times with isooctane and once with 40–60° light petroleum. The purpose of the latter was to remove most of the residual isooctane; the residual light petroleum, being much more volatile, was then driven off by shaking the flasks for 20 min at 37°.

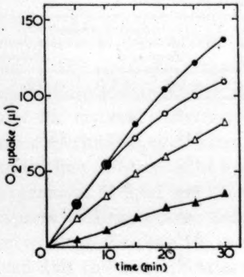

Fig. 4. Effect of extraction on added cytochrome *c* requirement of succinic oxidase. ●—●, unextracted plus added cytochrome *c*, $4.3 \cdot 10^{-5}$ *M*; △—△, unextracted without added cytochrome *c*; ○—○, extracted (once with 40–60° light petroleum using 1 ml preparation and 3 ml solvent) plus added cytochrome *c*, $4.3 \cdot 10^{-5}$ *M*; ▲—▲, extracted without added cytochrome *c*. Type II preparation suspended in Tris–HCl buffer. Residual solvent removed and succinic oxidase assayed as described in text.

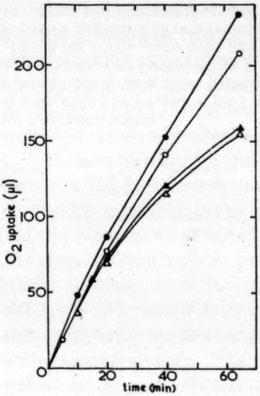

Fig. 5. Effect of *d*-α-tocopherol and ubiquinone additions on succinic oxidase of extracted preparation. ●—●, unextracted; △—△, extracted (four times with isooctane and once with 40–60° light petroleum using 1 ml preparation and 3 ml solvent); ○—○, extracted plus *d*-α-tocopherol (ethanolic solution), $2 \cdot 10^{-4}$ *M*; ▲—▲, extracted plus ubiquinone (ethanolic solution), $2 \cdot 10^{-4}$ *M*. Type II preparation suspended in Tris–HCl buffer. Residual solvent removed and succinic oxidase assayed as described in text.

Although there were no significant differences in the maximal activities, it will be noticed that *d*-α-tocopherol prevented the falling-off in activity with time. It seems that tocopherol is acting here as an antioxidant and is protecting some essential groups or components from destruction by oxidation. Tocopherol showed a similar effect with the unextracted enzyme preparations. AMES AND RISLEY[18] showed that although a suspension of α-tocopherol in bovine serum albumin inhibited the succinic oxidase activity of their preparations, it protected the system against inactivation for approx. 3 h. More recently, TAPPEL AND ZALKIN[19] found that tocopherol inhibited lipid peroxidation and stabilized DPNH–cytochrome *c* reductase in mitochondria.

Ubiquinone (*coenzyme Q*) *addition*: In a number of experiments, preparations of type II were extracted with isooctane and 40–60° light petroleum. The final extraction was usually made with light petroleum since this was more volatile and the residual solvent could be more readily driven off by incubating at 37° for relatively short periods (up to 20 min). The succinic oxidase activity could then be measured in the presence and absence of added ubiquinone (in ethanolic solution) and cytochrome *c*. The results (Table III) may be summarized as follows:

TABLE III

EFFECT OF ADDITION OF UBIQUINONE AND CYTOCHROME *c* ON SUCCINIC OXIDASE ACTIVITY OF SOLVENT-EXTRACTED PIG HEART-MUSCLE PREPARATIONS

All experiments were performed with type II preparations. Residual solvent removed and succinic oxidase assayed as described in text. Ubiquinone added where indicated (± UQ) as a ethanolic solution to give final concentration of $2 \cdot 10^{-4}$ *M*. Same volume of ethanol added to controls. Details of individual experiments as follows: Expt. 1: 2 ml preparation extracted once with 2 ml isooctane and then once with 2 ml 40–60° light petroleum. Expt. 2: 1 ml preparation extracted once with 3 ml isooctane and then once with 3 ml 40–60° light petroleum. Expt. 4: 1 ml preparation extracted once with 3 ml 40–60° light petroleum. Expt. 5: 1 ml preparation extracted twice with 3 ml isooctane and then once with 3 ml 40–60° light petroleum. Expt. 6: 2 ml preparation extracted with 4 ml 40–60° light petroleum.

Expt. number	*Preparation number*	*Age at time of Expt. (days)*	*Suspending buffer*	*Presence (+) or absence (—) of added cytochrome c*	*Succinic oxidase (QO_2)*			
					Unextracted		*Extracted*	
					— UQ	*+ UQ*	*— UQ*	*+ UQ*
1	31 B	6	Tris	—	128	129	58	50
	31 B	6	Tris	+	248	—	253	—
2	28 A	6	Phosphate	+	480	486	468	486
3	28 B	8	Tris	+	244	267	—	—
4	28 B	12	Tris	—	93	93	47	41
				+	152	—	152	—
5	25 B	2	Tris	+	278	—	221	226
6	29 A	2	Phosphate	—	76	51	41	41

(a) when cytochrome *c* was omitted, there was a loss of succinic oxidase activity after extraction; (b) in the presence of added cytochrome *c*, succinic oxidase activities either increased (*e.g.* to 22 % higher after two extractions) or decreased (*e.g.* 20 % lower after three extractions); (c) Ubiquinone added to the extracted preparation appeared to produce small increases in activity in two experiments; in most cases however, there were no significant changes in activity when ubiquinone was added with or without added cytochrome *c* except that it sometimes tended to prevent the falling-off in oxygen uptake with time. This effect may explain the slightly higher activities sometimes obtained with added ubiquinone. (d) Addition of ubiquinone to the unextracted preparation appeared to have little or no effect.

Effect of solvent-treatment on cytochrome oxidase

Extraction with non-polar solvents such as 40–60° light petroleum usually produced a marked increase in activity after removal of residual solvent as shown in Table II. It is known that surface-active agents have an activating effect on cytochrome oxidase[20] and it seems that non-polar solvents behave similarly.

When polar solvents are used, however, the results are rather different. In an experiment where a preparation was first extracted with isooctane, then with 40–60° light petroleum and finally with diethyl ether it was found that there had been a considerable inactivation of cytochrome oxidase and it had become the rate-limiting step in the succinic oxidase system. In another experiment in which a preparation was extracted once with 40–60° light petroleum containing 20 % (v/v) acetone the cytochrome oxidase was completely inactivated but succinic cytochrome *c* reductase was still relatively high. Thus it seems that treatment with non-polar solvents stimulates cytochrome oxidase activity but polar solvents produce an inactivation.

DISCUSSION

The heart-muscle preparations of the types described in this paper are colloidal suspensions of fragments of mitochondrial membranes and *cristae*. They contain 30–35 % lipid on a dry weight basis. Approx. 90 % of this lipid is phospholipid with smaller amounts of cholesterol (3–4 %), neutral lipid (2–3 %), ubiquinone (0.6 %) and other minor constituents[21]. There is a substantial amount of evidence to support the view that the phospholipid[22] is an important structural element, and the ubiquinone[23, 24] a functional component of the respiratory chain. Hence, removal of these lipids by extraction with organic solvents would be expected to have a profound effect on the enzymic activities of the system. However, the inactivation of the enzyme systems of the respiratory chain which occurs when heart-muscle preparations are treated with non-polar organic solvents appears to be due primarily to the inhibitory effect of the residual solvent present, and not to the removal of the lipid. In fact, in some experiments even after forty extractions there did not appear to be a loss in activity due to removal of lipid *per se*. In general, however, some loss in activity due to removal of lipid can usually be achieved after a sufficiently large number of extractions with non-polar solvents. Under the conditions usually employed by earlier workers (*i.e.* one or two extractions with isooctane) very little lipid was removed and the activity after removal of the residual solvent was usually higher than the original activity. The increase in activity which occurs after a few extractions can probably be attributed to the physical action of the solvent in breaking down larger particles or aggregates into smaller ones, or causing an alteration of the particle structure which allows an easier access of the enzyme system to the reactants. This process is probably analogous to the effect of freezing and thawing or to the action of certain surface-active agents, both of which result in increased enzyme activities. To summarize therefore, treatment of heart-muscle preparations with organic solvents has three principal effects: (a) the physical action of the solvent producing a change in particle size or morphology, (b) the removal of lipid from the particle by solution in the solvent, and (c) the retention of a small amount of the solvent by the particles by surface adsorption or by solution in the lipid. The observed loss in enzymic activities after one or two extractions with isooctane or other solvents is due primarily to the third effect. After removal of the inhibitory residual solvent the true activity of the extracted preparation can be measured. This may be higher or lower than the activity of the untreated control depending on the number of extractions and other factors such as the age and type of preparation.

It has been shown that a non-ionic surface active agent will effectively remove the inhibitory effect of the residual solvent presumably by dispersing it. It seems very likely that tocopherol, ubiquinone and other compounds with long isoprenoid side chains act similarly to surface active agents and are able to remove the solvent from its inhibitory site on the enzyme particle. WEITZEL *et al.*[25] have shown that tocopherol and vitamin K_1 exhibit surface-active properties. Compounds possessing long isoprenoid chains are however not the only ones which will reverse the inhibition due to solvent as recently pointed out by DRAPER AND CSALLANY[26].

In order to study the effect of various added lipids in lipid-extracted preparations it was necessary to devise another method for removing completely the residual solvent. This was achieved quite simply by first blowing a stream of nitrogen over the preparation at room temperature for a few minutes and then incubating for a short period at 37° to remove the last traces of solvent. Using this technique the effect of ubiquinone addition to extracted preparations was tested but in no case could an unequivocal activation by replacement be demonstrated. Thus although it seems probable from other work[23, 24] that ubiquinone is a functional member of the respiratory chain, under the conditions of the present experiments, added ubiquinone cannot restore activity by replacing extracted ubiquinone. CRANE *et al.*[27] extracted ETP with isooctane and found that succinic oxidase was partially reactivated with ubiquinone alone, completely reactivated by ubiquinone together with a special phospholipid fraction and completely reactivated by cytochrome *c* alone. There was no summation of the activities induced by ubiquinone and cytochrome *c*. These workers also found that although succinic oxidase activity fell after extraction succinic- and DPNH-cytochrome *c* activities increased markedly. It has been shown subsequently that ubiquinone acts before cytochrome *c* (see ref. 23) and its removal would, therefore, be expected to affect cytochrome reduction. Thus it is now very doubtful whether the reactivation obtained by these workers was due to a specific action of ubiquinone. The most likely explanation is that it was due to the removal of traces of solvent from the preparation. The explanation of the reactivating effect of cytochrome *c* is that treatment with organic solvents induces a requirement for added cytochrome *c* as already indicated in the present experiments. Recently the same group of workers[28, 29] have reported a specific reactivating effect of ubiquinone in acetone-extracted mitochondria. In this case the reactivation was only obtained when cytochrome *c* was also added; no reactivation was obtained with cytochrome *c* alone. It is difficult to understand why ubiquinone cannot reactivate preparations in which ubiquinone has been removed by extraction with non-polar solvents such as light petroleum while reactivations are obtained after much more drastic treatment with acetone. The explanation may be that the enzyme particles have to undergo some structural change only brought about by more polar solvents such as acetone, in order that the added ubiquinone can enter the active site. In support of this it has now been shown by CHANCE AND REDFEARN[30] that pure ubiquinone added to unextracted preparations is reduced at a very low rate compared with that of the endogenous material. An investigation of the mechanism of a specific reactivation by ubiquinone is in progress.

Finally, it is appropriate to mention the recent work of WEBER AND WISS[31]. These workers have shown that the inhibition of succinic–cytochrome *c* reductase activity in a mitochondrial preparation by isooctane and a number of other hydrocarbons is due not only to their lipid solubility but also to their structural properties. The length of the hydrocarbon chain was an important factor, chains of 6–7 carbon atoms being the most active. The inhibitory effect of the *n*-alkanes was decreased by the introduction of a hydroxyl group but was increased by bromine substitution. Of the other compounds with inhibitory effects it was interesting to note that a vitamin K_2 analogue with a side-chain consisting of one isoprenoid unit was 30 times as active as isooctane. In all cases the inhibition could be reversed by substances with long isoprenoid side chains such as vitamin K_1, phytol and ubiquinone. The potent inhibition caused by the vitamin K_2 analogue with a short side chain suggests a mechanism for the inhibition process. The short lipophilic side chain dissolves in the lipid phase of the enzyme particle leaving the large naphthoquinone nucleus projecting from the surface and acting as an efficient barrier to the reactants.

The structural similarity of the vitamin K_2 analogue and the 2-hydroxy-3-alkylnaphthoquinone inhibitors described by BALL, ANFINSEN AND COOPER[32] is striking. It is possible that the inhibitory effects of the latter can now be explained in terms of a purely physical non-specific effect rather than as a specific inhibition of a naturally occurring quinone, as recently suggested[33]. Work in progress may help to elucidate this point.

NOTE ADDED IN PROOF

After this paper was submitted for publication, a paper by HENDLIN AND COOK[34] has come to our notice. These workers have put forward evidence which supports the suggestion that naphthoquinones act as non-specific inhibitors.

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REFERENCES

[1] A. NASON AND I. R. LEHMAN, *Science*, 122 (1955) 19.
[2] A. NASON AND I. R. LEHMAN, *J. Biol. Chem.*, 222 (1956) 511.
[3] K. O. DONALDSON AND A. NASON, *Proc. Natl. Acad. Sci. U.S.*, 43 (1957) 364.
[4] G. V. MARINETTI, J. ERBLAND, M. MORRISON AND E. STOTZ, *J. Am. Chem. Soc.*, 80 (1958) 402.
[5] K. O. DONALDSON, A. NASON AND R. H. GARRETT, *J. Biol. Chem.*, 233 (1958) 572.
[6] D. DEUL, E. C. SLATER AND L. VELDSTRA, *Biochim. Biophys. Acta*, 27 (1958) 133.
[7] E. R. REDFEARN AND A. M. PUMPHREY, *Biochim. Biophys. Acta*, 30 (1958) 437.
[8] F. WEBER, U. GLOOR AND O. WISS, *Helv. Chim. Acta*, 41 (1958) 1038, 1046.
[9] C. J. POLLARD AND J. G. BIERI, *Biochim. Biophys. Acta*, 30 (1958) 658.
[10] D. KEILIN AND E. F. HARTREE, *Biochemical Preparations*, Vol. 2, 1952, p. 1.
[11] R. A. MORTON, U. GLOOR, O. SCHINDLER, G. M. WILSON, L. H. CHOPARD-DIT-JEAN, F. W. HEMMING, O. ISLER, W. M. F. LEAT, J. F. PENNOCK, R. RÜEGG, U. SCHWIETER AND O. WISS, *Helv. Chim. Acta*, 41 (1958) 2343.
[12] M. MORRISON, R. B. CRAWFORD AND E. STOTZ, *Biochim. Biophys. Acta*, 22 (1956) 579.
[13] A. M. PUMPHREY AND E. R. REDFEARN, *Biochem. J.*, 76 (1960) 61.
[14] K. W. CLELAND AND E. C. SLATER, *Biochem. J.*, 53 (1953) 547.
[15] E. R. REDFEARN AND A. M. PUMPHREY, *Biochem. J.*, 76 (1960) 64.
[16] R. B. CRAWFORD, M. MORRISON AND E. STOTZ, *Biochim. Biophys. Acta*, 33 (1959) 543.
[17] R. P. IGO, B. MACKLER AND D. J. HANAHAN, *J. Biol. Chem.*, 234 (1959) 1312.
[18] S. R. AMES AND H. A. RISLEY, *Ann. N.Y. Acad. Sci.*, 52 (1949) 149.
[19] A. L. TAPPEL AND H. ZALKIN, *Arch. Biochem. Biophys.*, 80 (1959) 333.
[20] W. W. WAINIO AND M. ARONOFF, *Arch. Biochem. Biophys.*, 57 (1955) 115.
[21] A. M. PUMPHREY AND E. R. REDFEARN, unpublished work.
[22] S. W. EDWARDS AND E. G. BALL, *J. Biol. Chem.*, 209 (1954) 619.
[23] A. M. PUMPHREY AND E. R. REDFEARN, *Biochem. J.*, 72 (1959) 2 P.
[24] D. E. GREEN AND R. L. LESTER, *Federation Proc.*, 18 (1959) 987.
[25] G. WEITZEL, A. M. FRETZDORFF AND S. HELLER, *Z. physiol. Chem., Hoppe Seyler's*, 303 (1956) 14.
[26] H. H. DRAPER AND A. S. CSALLANY, *Biochim. Biophys. Acta*, 38 (1960) 161.
[27] F. L. CRANE, C. WIDMER, R. L. LESTER AND Y. HATEFI, *Biochim. Biophys. Acta*, 31 (1959) 476.
[28] R. L. LESTER AND S. FLEISCHER, *Arch. Biochem. Biophys.*, 80 (1959) 470.
[29] F. L. CRANE, N. FECHNER AND K. S. AMBE, *Arch. Biochem. Biophys.*, 81 (1959) 277.
[30] B. CHANCE AND E. R. REDFEARN, in preparation.
[31] F. WEBER AND O. WISS, *Helv. Chim. Acta*, 42 (1959) 1292.
[32] E. G. BALL, C. B. ANFINSEN AND O. COOPER, *J. Biol. Chem.*, 168 (1947) 257.
[33] C. D. JOEL, M. L. KARNOVSKY, E. G. BALL AND O. COOPER, *J. Biol. Chem.*, 233 (1958) 1565.
[34] D. HENDLIN AND T. M. COOK, *Biochem. Biophys. Res. Comm.*, 2 (1960) 71.

THE STRUCTURE OF CHITIN

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SUMMARY

A new structure is proposed for the polysaccharide chitin, $[C_8H_{13}O_5N]_n$. X-ray diffraction fibre photographs of lobster tendon reveal a well-defined orthorhombic lattice with the space group $P2_12_12_1$ and cell dimensions of $a = 4.69$ Å, $b = 19.13$ Å, and $c = 10.43$ Å (fibre repeat). It is suggested that the unit cell contains two polysaccharide chains running in opposite directions and four asymmetric N-acetylglucosamine units which gives a density in close agreement with the observed experimental value of 1.42 g/ml. The structure was arrived at by a trial and error method, with the aid of an optical diffractometer for testing the diffraction patterns of the various possibilities suggested by stereochemical considerations. The structure finally arrived at, is in good agreement with infra-red absorption data, and has no short contacts. The chains are separated by a distance of 4.69 Å perpendicular to the "plane" of the sugar rings and consequently the NH and CO groups in the neighbouring aminoacetyl side chains are hydrogen bonded, the plane of the amide groups in the "trans" configuration being almost perpendicular to the fibre axis. The hydroxymethyl side chains containing a hydroxyl group are also hydrogen bonded, the OH of one being linked to the oxygen of a similar group from a neighbouring chain running in the opposite direction. The short hydroxyl group is intrahydrogen bonded to the oxygen of the amide group in the same asymmetric unit. The calculated intensities for the proposed set of coordinates are in fairly good agreement with observed X-ray intensities visually estimated from fibre photographs.

INTRODUCTION

Chitin, like cellulose, is a straight chain polysaccharide and is a condensation product of N-acetylglucosamine with the formula $[C_8H_{13}O_5N]_n$; it occurs both as well oriented fibres, as in the mandibular tendon of lobster, and in the form of sheets, as in the cuticles of arthropods. From X-ray diffraction photographs of lobster tendon MEYER AND PANKOW[1] deduced that the unit cell is orthorhombic, with $a = 9.40$ Å, $b = 10.46$ Å (fibre repeat) and $c = 19.25$ Å. Observing odd order meridional reflections in their normal beam diffraction photographs, they rejected the possibility of a two-fold screw axis along the fibre axis. Further, from the absence of a first order reflection of 19.25 Å, and also the possible absence in general of $00l$ with l odd, they were led to the two possible space groups, namely $P222_1$, and $P2_122_1$ for the structure. Of these, MEYER AND PANKOW[1] favoured the latter, which was also confirmed by

Darmon and Rudall[2] from their x-ray studies on doubly-oriented cuticles with the $\{00l\}$ planes lying nearly parallel to the surface, which definitely revealed the absence of the 100 reflection of spacing 9.40 Å.

Postulating an analogy between cellulose and chitin, and using the known chemical configuration of N-acetylglucosamine, Meyer and Mark[3]—also Meyer and Wehrli[4]—arrived at the spatial formula for chitin in which the pyranose rings are linked by a 1,4 type of bonding (Fig. 1). This was confirmed by the work of Bergmann, Zervas and Silberweit[5]. Meyer and Pankow[1] arranged the chains thus built up in their lattice so that the chains were separated by about 9.6 Å along *c* in the direction of the plane of the sugar rings, and by about 4.65 Å along *a* in the direction perpendicular to them. With this arrangement of the chains conforming to the space group $P2_122_1$ there are 4 chains, and therefore 8 pyranose rings, contained in the unit cell. However, the space group requires only 4 equivalent points in the general position. To overcome this difficulty Meyer and Pankow[1] introduced the concept of the chitobiose unit, analogous to the cellobiose unit in cellulose, in which they postulated that the two adjacent pyranose rings along a chain, which together formed a fibre period of 10.46 Å along the *b* axis, were similar but not crystallographically identical in that they do not pass into each other by any symmetry operation. Then, the four chitobiose units which occurred in the unit cell of the above dimensions led to a calculated density value of 1.4 g/ml which agrees well with the measured value of 1.415 g/ml.

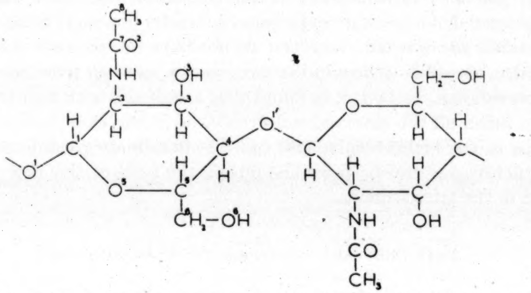

Fig. 1. The spatial configuration of side groups in chitin showing the two pyranose rings which constitute the repeating unit along the fibre axis.

Rudall[6] suggested that due to the presence of the amino-acetyl side chains of chitin, C=O . . . H–N hydrogen bonds could be formed of the type common in proteins. In attempting to make hydrogen bonds of this kind between amide groups of adjacent chains, Rudall[6] found two possible different types of arrangement depending on whether the amide groups were in the *trans* or *cis* configuration. He then postulated that both configurations were present in the structure depending upon the positioning of the side groups with respect to the 2-fold and screw axis elements in the lattice. Rudall[6] believed that this was supported by the interpretation of the infra-red absorption data obtained by Darmon and Sutherland[7]. This does not, however, seem to be necessarily true (see discussion (b)).

It is commonly found in most high polymers occurring in a crystalline form that the residues or units in a chain take up crystallographically equivalent positions, either on an integral or non-integral helix. It was therefore rather surprising that the two pyranose rings in the chitin chain were not equivalent crystallographically in MEYER AND PANKOW's structure[1]. It is of course not unusual for the unit cell of a crystal to contain more than one set of equivalent groups as in this case (8 units, forming two sets of four equivalent units). However, there appeared to be a *prima facie* case for a re-examination of the structure owing to this feature, and further scrutiny has justified the doubt. Thus, the absence of a 2-fold screw axis along the fibre (*b*) axis was deduced from the occurrence of weak horizontal streaks along the meridian at positions corresponding to the odd-order reflections. This deduction is, however, not valid, for such streaks joining nearly non-meridional reflections are found in various fibres which, however, do not have an intensity peak at the exact meridian. Also, as will be shown in the next section, the 030 reflection can be proved to be non-meridional. In fact, it is found that a unit cell with half the above size is sufficient to index all the observed reflections and in this case there are just 4 equivalent groups in the orthorhombic unit cell. The details are given in the next section. The full structure has also been worked out on the basis of this new unit cell and it is discussed in the later sections.

UNIT CELL AND SPACE GROUP DETERMINATION

X-ray diffraction photographs of fibrous chitin, obtained from the mandibular tendon of a lobster, were taken with a micro-camera with a very fine collimator,

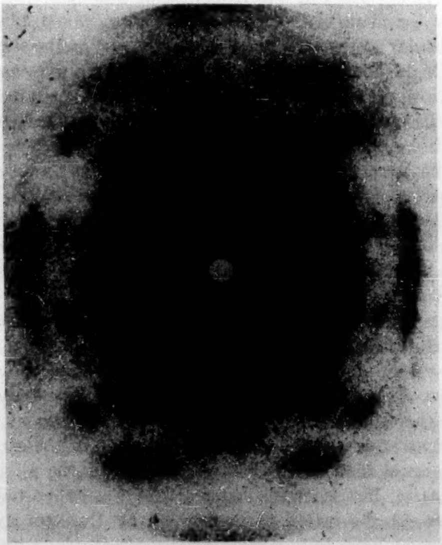

Fig. 2. X-ray diffraction photograph of chitin (enlarged to correspond to D = 4 cm) with X-ray beam normal to fibre axis.

using Ni filtered Cu $K\alpha$ radiation and with a fibre-film distance of 1.5 cm (flat film technique). This is shown in Fig. 2. The fibre was purified by treating it in the cold with dilute caustic potash, and then with dilute hydrochloric acid, each for about half an hour. It was then washed thoroughly in distilled water and dried with ether.

The Bragg spacings d of all the observed reflections were accurately measured for all the layers and are tabulated in Table I. From the spacings, the values of $(d^*)^2 = \lambda^2/d^2$ were calculated for all reflections. The fibre repeat c was measured as a mean of the layer-line spacings of the various observed layers and led to a value of $c = 10.43$ Å. Using this, the ξ^2 value of each reflection was calculated from the formula $\xi^2 = (d^*)^2 - (l\lambda/c)^2$. All these values are tabulated in Table I.

By a method of trial and error the smallest possible unit cell was deduced. As mentioned above, the spacings of the layer lines gave directly the fibre repeat $c = 10.43$ Å. The other two spacings a and b were obtained by trial and error from the

TABLE I

DATA OBTAINED FROM MICRO-CAMERA PHOTOGRAPHS

	Bragg spacing d (Å)	*Observed* d^{*2}	*Observed* ξ^2	*Calculated* ξ^2	*Indices* $h\,k\,l$	*Observed intensity* I_0
Equator	9.542	0.026	0.026	0.0260	020	19
	4.595	0.113	0.113	0.1145	110	100
	4.257	0.131	0.131	0.1340	120	0.5
	3.761	0.168	0.168	0.1665	130	12.5
	3.328	0.215	0.215	0.2120	140 (060)*	2
	2.311	0.445	0.445	0.4385	210 (200, 220)	12
First layer	9.071	0.029	0.007	0.0065	011	1
	6.940	0.049	0.027	0.0260	021	4
	4.257	0.131	0.109	0.1080	101 (041, 111)	7
	3.914	0.155	0.133	0.1340	121	1
	3.183	0.235	0.213	0.2120	141	2.5
	2.820	0.299	0.277	0.2705	151 (071)	1
	2.544	0.367	0.345	0.3420	161	2.5
	2.288	0.454	0.432	0.4320	201 (211, 171)	6
Second layer	5.056	0.093	arc	—	002 (012)	12
	4.514	0.117	0.026	0.0260	022	0.5
	3.970	0.151	0.060	0.0585	032	4
	3.445	0.200	0.110	0.1080	102	6
	3.031	0.259	0.170	0.1665	132	5
	2.580	0.357	0.269	0.2705	152 (072)	1.5
	2.157	0.511	0.423	0.4265	172 (082)	1
Third layer	3.358	0.211	arc	—	013**	50
	3.157	0.239	0.028	0.0260	023	3
	2.772	0.310	0.113	0.1145	113 (103)	6
	2.544	0.367	0.170	0.1665	133 (053)	6
	2.237	0.475	0.278	0.2705	153 (063)	1.5
Fourth layer	2.237	0.475	0.109	0.1080	104 (114)	2.5
	2.133	0.523	0.173	0.1665	134 (054)	1

* Other possible indices for the reflections are also given in brackets.

** Was split into two non-meridional reflections in the inclined fibre photograph.

equation $\xi^2 = h^2a^{*2} + k^2b^{*2}$. The task was comparatively easy, since trial values of a and b were taken to be $b = 19.25$ Å, corresponding to the spacing of the structure of MEYER AND PANKOW[1], and $a = 4.7$ Å which is half of MEYER AND PANKOW's value for a. The final dimensions were $a = 4.69$ Å, $b = 19.13$ Å and $c = 10.43$ Å (fibre repeat). (Note the change in designation of the axes from that adopted by MEYER AND PANKOW[1].)

There is, however, one very faint reflection present in the general equatorial streak in the diffraction photographs with spacing $d = 5.51$ Å, which does not fit in with this unit cell (or with the one given by MEYER AND PANKOW[1]). Consequently it has been omitted, as it is probably due to some radiation artifact or some impurity in the fibre used, which cannot be easily removed. Incidentally, this very weak reflection has not been observed by MEYER AND PANKOW.

Thus the unit cell is half that given by MEYER AND PANKOW. Consequently the space-group has also to be redetermined. There seems to be clear evidence for the presence of a two-fold screw axis parallel to the fibre axis. This means that odd order meridional reflections should be absent. Now the first order meridional reflection is very, very faint and is in fact a long streak bridging the $\{011\}$ reflections on either side of the meridian. This type of streak does not indicate the existence of a meridional reflection unless there is an intensification at the meridian, for it could arise from the occurrence of a cylindrical lattice (as is found in the 9-Å layer of collagen) or from disorder, etc. Actually, no intensification was found on the meridian even in a tilted fibre photograph.

Similarly, there is a very strong meridional arc on the third layer in the normal beam picture, which has to be explained. A tilted fibre X-ray diffraction photograph was therefore taken with the fibre inclined at the equi-inclination angle of the third layer, namely 13°. The photograph (Fig. 3) clearly showed that the arc is split up into two non-meridional reflections which, however, still overlap, with the indices 013. Similar tilted fibre photographs taken for the fourth and second layer equi-inclination angles revealed strong intensification of the meridional reflections 004 and 002. No fifth order meridional reflection 005 was observed in the former. Thus reflections of the type $00l$ with l odd seem to be absent and hence there must be a screw axis parallel to the fibre axis.

Considering next the other two 2-fold axes parallel to a and b, it is seen from Table I that among $h00$ only 200 probably occurs, while among reflections of the type $0k0$, 020 and 060 are recorded while 010, 030 and 050 seem to be absent. It is therefore reasonable to assume that there is a 2-fold screw axis along $b = 19.1$ Å, but along a the evidence is not clear since reflections with $h > 2$ are beyond the range of the observed diffraction pattern. Thus, the two possible space groups are $P22_12_1$, and $P2_12_12_1$, all space-groups containing reflection or glide planes being impossible since the compound is asymmetric.

The choice between the two space-groups was, however, readily made by considering the possible structural configurations. It will be seen that in the case of the space-group $P22_12_1$ (Fig. 4(a)), the chains running in opposite directions would be in the same plane parallel to the 19.1-Å axis, and also have the plane of their pyranose rings in this plane as well. As a result the side-chains belonging to the adjacent chains would either have to abut or interlock with each other. When the longer side-chains abut there will definitely be undesirable short contacts. When they are made to interlock the orientation of the chains are such that in no position do they give rise to suitable cross-chain hydrogen bonding between hydroxymethyl groups of adjacent chains parallel to the plane of the pyranose rings, as is obtained in Fig. 4(c) with the space group $P2_12_12_1$. Even if the chains are rotated about the screw axes parallel to them no suitable configuration is obtained for cross-chain hydrogen bonding. This cannot be achieved with the space group $P22_12_1$ as the pyranose rings are separated by 4.7 Å in a direction perpendicular to the plane of the rings. In these respects, the space group $P2_12_12_1$ is superior in that there is a position in which adjacent chains can be readily hydrogen bonded (as we shall see in a later section) to give a structure that is stereochemically very satisfactory. This comes about because of the fact that along the 19.1-Å direction, adjacent chains are not in the same plane but are staggered by a half-unit translation along the *a* axis. Finally it may be said that the absence of the 100 reflection is consistent with the space group $P2_12_12_1$ although this alone would not demand the space group assigned.

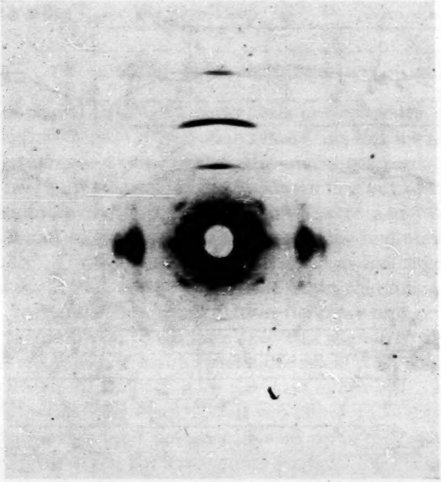

Fig. 3. X-ray diffraction photograph of chitin, with fibre inclined through the equi-inclination angle for the third layer namely 13° from the normal position of Fig. 2. Note the splitting of the meridional arc in the third layer and the occurrence of a meridional spot in the fourth layer.

Consequently, chitin possesses an orthorhombic structure with cell parameters $a = 4.70$ Å, $b = 19.1$ Å and $c = 10.4_3$ Å (fibre period), and with the space group $P2_12_12_1$, having four molecules per unit cell.

ESTIMATION OF INTENSITIES OF REFLECTIONS

To determine the intensities of the various reflections, X-ray fibre photographs of chitin were taken using a cylindrical camera of radius 3 cm, and Ni-filtered Cu $K\alpha$ radiation. The usual multiple film technique was adopted, although only two films were used, and the exposure was made with the fibre stationary. The intensities of the reflections on both films were then visually estimated with the aid of a set of standard spots which were recorded using the intense reflection 020 from the chitin fibre itself.

To obtain the integrated intensity of each reflection, the observed value of the peak intensity was multiplied by half the spread of the reflection along its corresponding powder ring.

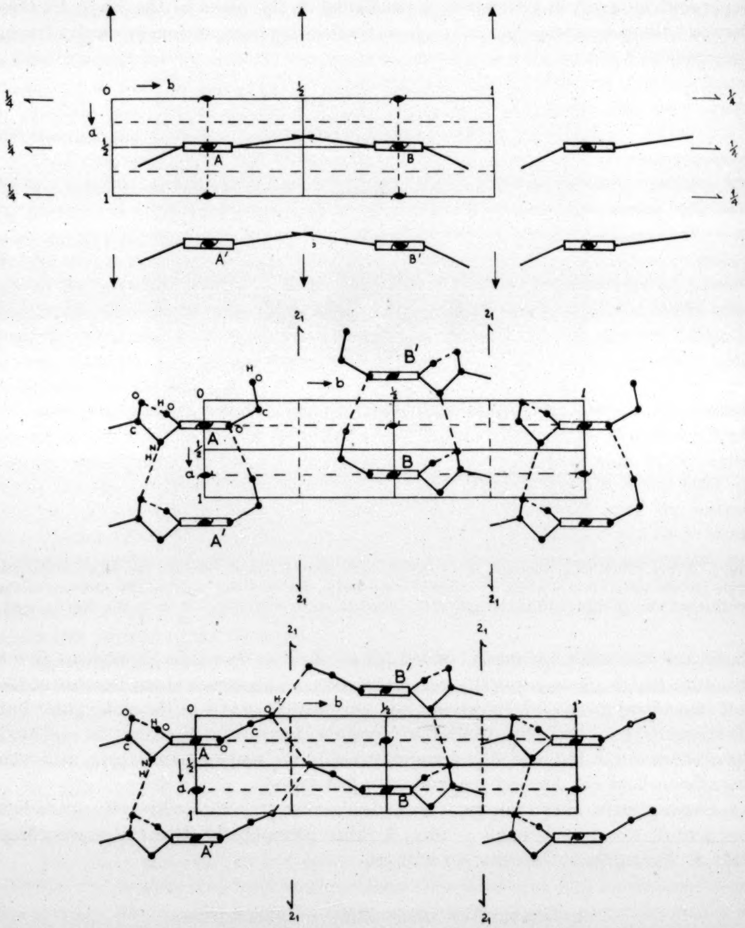

Fig. 4(a). A schematic c-projection of one half of the unit cell along the c-axis in the case of the space group $P22_12_1$; (b) A schematic c-projection of one half of the unit cell along the c-axis in the case of the space group $P2_12_12_1$ and with the parameter $u = 0$; (c) A schematic c-projection of one half of the unit cell along the c-axis in the case of the space group $P2_12_12_1$ and with the parameter $u = -0.25$.

These integrated intensities were then corrected by the Lorentz and polarization factors, which together are given for a general level by the expression

$$\frac{1 + \mathrm{Cos}^2\, 2\,\theta}{\mathrm{Sin}\, 2\,\theta} \cdot \frac{\mathrm{Cos}\,\theta}{[\mathrm{Sin}^2\,\alpha - \mathrm{Sin}^2\,\theta]^{1/2}}$$

where, α is the angle between the normal to the reflecting planes ($h\,k\,l$) and the fibre axis, and θ is the Bragg angle.

These corrected intensities were then suitably scaled to make the strongest observed reflection, namely 110 with $d = 4.59$ Å, equal to 100. These final values of the observed intensities I_0 are given in Table III.

DETERMINATION OF THE STRUCTURE

Models of the chitin chains were built up to scale in conformity with the chemical formula of chitin, and with the 1,4 type of pyranose ring linkage through an oxygen atom. The bond distances and bond angles were made in accordance with standard values, while the directions of some of the bonds coming out of the ring as well as the "chair" shaped configuration of the ring itself were maintained to be nearly the same as those found in the structure of glucosamine hydrobromide worked out by COX AND JEFFREY[8]. The side chains were attached as indicated by the chemical formula, while the orientation of the longer side chain containing the amide group was arranged such that the amide group held in the *trans* configuration was planar, and with its plane perpendicular to the fibre axis. This planarity of the amide group is now pretty well established, while the particular orientation was adopted since the infra-red absorption data of DARMON AND RUDALL[2] indicate it. The hydroxymethyl or shorter side group was not rigidly fixed but was allowed to assume any convenient orientation. The chains, thus built up, were then set up in the orthorhombic unit cell so as to satisfy the symmetry of the space group $P2_12_12_1$. The chains themselves were kept with their length along the screw axes 2_1 parallel to the c axis, which is required by its chemical structure (Fig. 1). Then, the other two 2-fold screw axes pass midway between the chains.

Fig. 4(b) shows schematically the projection of one half of the unit cell along the c axis. There are two chains in the unit cell running in opposite directions. If the chain nearer the origin of the lattice (A in Fig. 4(b)) is assumed to be coming up, the other near the middle of the cell (marked B) is going down. The chains A and A′ separated along the a-axis are similar, and are a unit translation apart. It was then possible to bond the amide groups of such adjacent chains, the N–H of one chain bonding with the C=O of a similar chain, the hydrogen bond distance being 2.8 Å, while the associated bond angle is within the usual range. The O–H group attached to the ring directly could not be bonded to any group in another adjacent chain. However, it could be internally hydrogen bonded with the oxygen atom of the amide group attached to the same chain, the O–H . . .O=C bond distance being within the permitted limit of 2.8 Å.

The bonding of the hydroxymethyl group was examined next. Now since the chains running in opposite directions parallel with the c axis have to coincide with the screw axes, the number of possible orientations for each chain is limited. The two possible degrees of freedom permitted for each chain are (1) a relative displacement along the screw axis parallel to the *c* axis, and (2) a (slight) rotation of the chains about this axis. The latter is limited since the *a*-parameter is short and a large rotation would cause the atoms of the long side-chains containing the amide groups to approach each other and cause short contacts. Consequently, rotations of the two chains were not considered and only the effect of displacements of the chains along the screw axes were examined in detail.

Initially the oxygen atom connecting two pyranose rings, namely O'_1, was adjusted to be in a special position, namely in the *ab* plane containing the origin. For convenience let this position be represented by a parameter $u = 0$, expressed as a fractional coordinate of the translation of the atom O'_1 along the positive *c* axis. Having thus fixed the position of chain A, the position of chain B was fixed by the operation of the screw axis parallel to the *a* axis. It was then found that the hydroxymethyl group of chain A could not hydrogen bond with the hydroxymethyl group of chain B. Bonding of this group was then tried between similar chains A and A′, and it was found that it was possible to bond the hydroxymethyl group of chain A′ with the oxygen atom in the ring of chain A as shown in Fig. 4(b). The O–H . . .O hydrogen bond distance was slightly too large, but it could be brought to a reasonable value by slightly distorting some bond angles. This arrangement was stereochemically not very satisfactory since unusual hydrogen bonding was encountered, and further

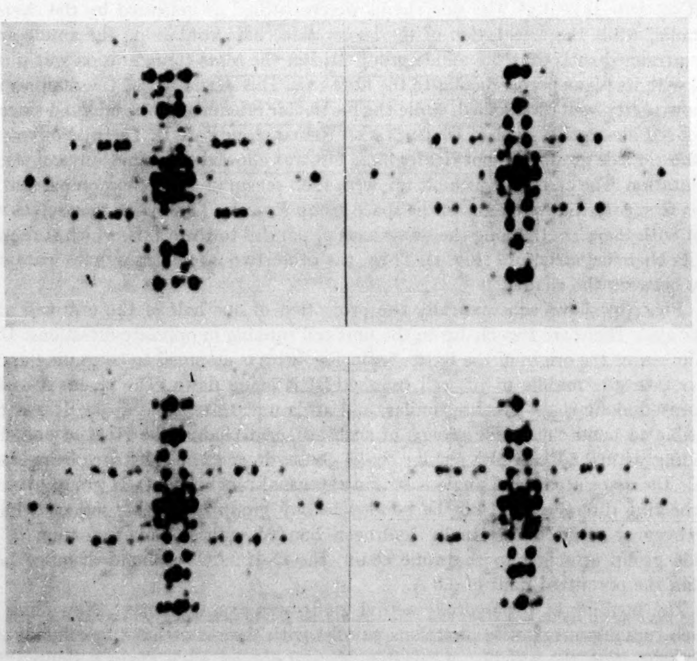

Fig. 5. Optical transforms of the *a*-projection of four unit cells of the structure having the parametres (a) $u = -0.08$; (b) $u = -0.14$; (c) $u = -0.16$; (d) $u = -0.18$.

there was no stabilizing cross-hydrogen bonding between chains A and B. However, the coordinates of all the carbon, nitrogen and oxygen atoms in one asymmetric unit were obtained as accurately as possible from the model. The intensities of all the reflections within the observed $\sin\theta$ range were calculated and compared with the observed intensities.

Fairly good agreement was obtained for the zero level, but for higher levels the agreement was far from good as large discrepancies between calculated and observed intensities were found. This indicated that in projection the structure was almost correct, *i.e.*, that the coordinates along the fibre axis were evidently in error while the x and y coordinates were almost correct. The correctness of the projection indicated that no rotation of the chains about the fibre axis was required and that a shift along the fibre axis was necessary for getting good agreement with higher layer data.

This shift was then applied to the chain A, the oxygen atom O'_1 being moved down along the negative direction of c by 0.25 of the translation c. This configuration is represented by the parameter $u = -0.25$, and is the maximum shift which must be considered, all possible configurations being contained in the range $0.25\ u$. Bonding between the hydroxymethyl groups of chain A and A′ with the hydroxymethyl group in chain B now became possible, the type of bonding being as shown in Fig. 4(c), where the O–H . . .O hydrogen bonds are inclined at about 45° to the planes of the sugar rings. The hydrogen bond angles are better than before though the bond distance is slightly large, around 2.9 Å. This structure is more satisfactory than the former in that cross hydrogen bonding between all chains is now possible. The intensities of all reflections within the observed range of $\sin\theta$ were again calculated and checked against the observed intensities. More satisfactory agreement was found for some levels though the agreement was not complete.

This indicated that the z coordinates were still in error, and a change in the parameter u was required. The parameter was therefore varied continuously between $u = 0$ and $u = -0{,}25$ and the condition for the most satisfactory hydrogen bonding of hydroxymethyl groups of adjacent chains was examined. It was found that hydrogen bonding was possible within fairly wide limits of translation of the chains, from about $u = -0.08$ to $u = -0.25$, with the best hydrogen bonding around $u = -0.14$. It was then decided to try out four different configurations represented by the parameters $u = -0.08$, $u = -0.14$, $u = -0.16$ and $u = -0.18$. Values of $|u| > 0.18$ were not considered as $u = -0.25$ did not give very satisfactory agreement with observed data.

In order to reduce the amount of work, optical transforms of the a-projection of the four different structures were taken. These were obtained using a diffractometer constructed in the laboratory. In this way all the general reflections hkl could not be obtained, but only reflections of the type $0kl$. However, these were sufficient to decide the correct position of the chains from among the four configurations decided upon. This was because most of the low angle reflections observed were of the type $0kl$, and also because many of them were in the high intensity group with clearly-marked ratios among their various individual intensities.

Four a-projection masks were prepared for the four different structures mentioned earlier; the optical transforms corresponding to the four values of u are shown in Fig. 5.

The transforms obtained with the parameters $u = -0.08$ and $u = -0.18$

indicated ratios among the intensities which were rather different from those in the x-ray diffraction photographs. For instance in Fig. 5(a) with the chains having the parameter $u = —0.08$, the third layer was very weak in the transform, whereas a strong x-ray reflection 013 is actually recorded. In addition to this the second and fourth order meridional reflections were also absent in the transform indicating that the structure was considerably in error. In the transform obtained with the parameter $u = —0.18$ (Fig. 5(d)) the general appearance was better in that the fourth order meridional reflection was observed, and also the reflection 013 was not too weak. Here again the ratios of the intensities were not quite correct, the intensity of 014 being too high. This was again an indication that the structure was in error.

In the transform obtained with the chains having the parameter $u = —0.16$ (Fig. 5(c)) the general appearance was better than for $u = —0.18$, in that 004 was stronger while 014 had decreased. Again 013 was stronger than before though not as strong as it should be, and the magnitude of the ratios among other reflections had also improved indicating that the correct configuration was a little lower probably at $u = —0.14$.

The transform with $u = —0.14$ (Fig. 5(b)) gave the best results of all, the agreement with observed x-ray diffraction data being good for all levels. The 014 reflection had almost fallen to zero, while the magnitude of 013 was the highest recorded over the entire range studied. The ratio of 011 to 021 was also fairly near the experimental value. The actual magnitudes of the different ratios, were, however, not quite correct indicating that while the positions of the main chain in the lattice were roughly correct, the coordinates of the atoms in the side chains required slight alteration.

An excellent feature of this configuration with parameter $u = —0.14$ was that the O–H . . .O hydrogen bonds between the hydroxymethyl groups of adjacent chains were the shortest possible (2.8 Å), and further the O-H group was also directed almost straight to the oxygen atom of the adjacent group.

Before calculating the intensities for all reflections, the amide group was tilted very slightly so as to improve the internal hydrogen bond distance to 2.6 Å. An optical transform taken with this minor change indicated slight improvement thus confirming the shorter bond distance.

Refinement of the structure was stopped at this stage and the calculation of intensities for all reflections was carried out. Since the agreement for the zero level was found to be good, no attempt was made to rotate the chains about the screw axis. Thus in the *c*-projection the plane of the rings is perpendicular to the *a* axis. The final fractional coordinates of one asymmetric unit are given in Table II omitting the hydrogen atoms. These final coordinates are expected to be accurate to about ± 0.5 Å for atoms in the side-groups and much less for atoms in the pyranose ring. A more accurate estimate of the coordinates, as well as the deviation of the various atoms in the molecule from these coordinates is not possible at this stage. Further refinements in the coordinates is possible by adopting the least square refinement techniques, but this is not likely to alter the sterochemical configuration of the present molecular arrangement.

COMPARISON OF CALCULATED AND OBSERVED INTENSITIES

The intensities of all reflections up to the fourth layer were calculated, so as to cover the observed range of sin θ using the standard formula for the space group $P2_12_12_1$

given in the International Tables for Crystallography and also the standard scattering factors for carbon, nitrogen and oxygen atoms given by VIERVOLL AND ÖGRIM[9]. In these calculations the contributions of the hydrogen atoms have been ignored. The calculated intensities were corrected for a temperature effect, by applying the usual temperature factor for intensities, namely $\exp - 2B(\sin \theta/\lambda)^2$. An isotropic temperature factor was assumed, and it was found that a value of $B = 1.5$ Å^2 agreed best with the observed decay of intensities with increasing Bragg angle. All the calculated intensities were then corrected for the temperature effect with this value, and these corrected intensities I_c are given in Table III.

TABLE II

DESCRIPTION OF THE STRUCTURE OF CHITIN; FRACTIONAL COORDINATES OF ATOMS IN ONE N-ACETYLGLUCOSAMINE RESIDUE

Unit Cell: Orthorhombic; $a = 4.69$ Å; $b = 19.13$ Å; $c = 10.43$ Å (fibre repeat). Space-group: $P2_12_12_1$. Set of equivalent points: x,y,z; $\frac{1}{2} - x, \bar{y}, \frac{1}{2} + z$; $\frac{1}{2} + x, \frac{1}{2} - y, \bar{z}$; $\bar{x}, \frac{1}{2} + y, \frac{1}{2} - z$.

Atom	*x*	*y*	*z*
C_1	0.324	0	0.232
C_2	0.223	— 0.063	0.190
C_3	0.276	— 0.063	0.046
C_4	0.164	0	— 0.002
C_5	0.250	0.063	0.046
C_6	0.084	0.136	0
C_7	0.170	— 0.175	0.201
C_8	0.255	— 0.251	0.254
N	0.383	— 0.120	0.206
O_1	0.250	0	0.360
O_2	— 0.044	— 0.167	0.179
O_3	0.155	— 0.107	— 0.077
O_5	0.250	0.063	0.190
O_6	0.250	0.200	0

The intensities I_c were then scaled so that the most intense reflection, namely 110 with spacing 4.59 Å, had a value 100. The other calculated intensities were similarly scaled, and these values I_c^* are also given in Table III.

From the sin θ values in Table III it will be noticed that many calculated reflections *hkl* have Bragg angles quite close to one another. Since in x-ray diffraction photographs of fibres, the reflections are diffuse and have a definite spread of intensity, allowance has to be made for the possible overlap of reflections. Consequently, the intensities of overlapping reflections have been added together wherever it occurs. These total intensities have also been given in the column I_c^*, bracketing those reflections which have contributed to the sum. Comparison of calculated and observed intensities indicate good agreement for all levels.

However, a few near-meridional reflections are still found to have a larger observed intensity than the values calculated for them. This phenomenon must be attributed to the fact that the fibres have a small spread in their orientation with respect to the fibre axis. Since the Lorentz factor is very sensitive to small changes in ξ near the meridian (actually $\rightarrow \infty$ for $\xi = 0$), the observed intensity will be larger,

TABLE III

CALCULATED AND OBSERVED INTENSITIES

	h k o	*Calculated sin θ*	I_c	I_{c^*}		*Observed sin θ*	I_o
Zero layer	020	0.081	4790	21.0		0.081	19.0
	040	0.160	29	0			
	110	0.168	22802	100.0		0.168	100.0
	120	0.181	135	0.6		0.181	0.5
	130	0.205	481	2.1		0.205	12.5
	140	0.232	213	0.9			
	060	0.240	243	1.1	1.7	0.240	2.0
	150	0.259	128	0.6			
	160	0.291	68	0.3			
	080	0.321	0	0			
	170	0.325	78	0.3			
	200	0.329	3061	13.4	15.9	0.334	12.0
	210	0.334	14	0.1			
	220	0.339	548	2.4			
	230	0.350	104	0.5	2.9	0.365	2.5
	180	0.360	12	0			
	240	0.366	539	2.4			
	250	0.385	28	0.1			
	190	0.396	46	0.2	1.1	0.408	1.5
	0100	0.401	52	0.2			
	260	0.407	76	0.3			
	1100	0.429	82	0.4			
	270	0.432	0	0			
	280	0.459	21	0.1			
	290	0.488	19	0.1			
	h k 1	*Calculated sin θ*	I_c	I_{c^*}		*Observed sin θ*	I_o
First layer	011	0.085	189	0.8		0.085	1.0
	021	0.111	1639	7.2		0.111	4.0
	031	0.141	491	2.2			
	041	0.177	1772	7.8	12.2	0.181	7.0
	101	0.181	7	0			
	111	0.185	998	4.4			
	121	0.197	372	1.6		0.198	1.0
	051	0.214	63	0.3			
	131	0.217	263	1.2			
	141	0.242	356	1.6		0.242	2.5
	061	0.252	69	0.3			
	151	0.273	4	0			
	071	0.290	177	0.8		0.280	1.0
	161	0.303	524	2.3		0.305	2.5
	081	0.329	194	0.9	4.0	0.338	6.0
	171	0.333	130	0.6			
	201	0.337	187	0.8			
	211	0.340	235	1.1			
	221	0.347	131	0.6			
	231	0.358	59	0.3			
	181	0.368	188	0.8		0.368	1.0
	091	0.368	7	0			
	241	0.373	21	0.1			
	251	0.392	19	0.1	0.4	0.403	1.0
	191	0.403	33	0.2			
	0101	0.407	13	0.1			
	1101	0.439	3	0			

TABLE III (continued)

	$h\,k\,2$	Calculated $\sin\theta$	I_c	I_{c^*}		Observed $\sin\theta$	I_o
Second layer	002	0.148	197	0.9	4.3	0.148	12.0
	012	0.153	777	3.4			
	022	0.168	27	0.1		0.170	0.5
	032	0.191	709	3.1		0.194	4.0
	042	0.218	72	0.3	1.5	0.224	6.0
	102	0.221	136	0.6			
	112	0.225	32	0.2			
	122	0.236	97	0.4			
	052	0.249	56	0.2	3.0	0.255	5.0
	132	0.252	381	1.7			
	142	0.273	251	1.1			
	062	0.282	12	0.1			
	152	0.299	68	0.3			
	072	0.317	417	1.8		0.310	1.5
	162	0.327	314	1.4		0.330	1.5
	082	0.353	12	0.1			
	172	0.357	240	1.1		0.357	1.0
	202	0.361	11	0			
	212	0.363	8	0			
	222	0.370	142	0.6	6.1	0.390	4.0
	232	0.380	16	0.1			
	182	0.389	483	2.1			
	092	0.390	743	3.3			
	242	0.395	23	0.1			
	192	0.423	14	0.1			
	0102	0.427	99	0.4			
	1102	0.458	119	0.5			

	$h\,k\,3$	Calculated $\sin\theta$	I_c	I_{c^*}		Observed $\sin\theta$	I_o
Third layer	013	0.226	2831	12.4		0.226	50.0
	023	0.236	1095	4.8		0.240	3.0
	033	0.252	37	0.2			
	043	0.274	12	0.1	3.1	0.278	6.0
	103	0.276	54	0.2			
	113	0.279	453	2.0			
	123	0.288	183	0.8			
	053	0.299	882	3.9	6.0	0.303	6.0
	133	0.301	104	0.5			
	143	0.319	365	1.6			
	063	0.327	205	0.9	1.8	0.345	1.5
	153	0.341	69	0.3			
	073	0.358	145	0.6			
	163	0.366	9	0			
	083	0.390	10	0			
	173	0.394	127	0.6			
	203	0.397	118	0.5			

even after correcting for the Lorentz factor corresponding to the mean value of ξ. If allowance is made for this fact, the agreement between observed and calculated intensities can be considered to be fairly good even for these reflections.

TABLE III (continued)

	$hk4$	Calculated $\sin\theta$	I_c	I_{c^*}		Observed $\sin\theta$	I_o
Fourth layer	004	0.296	1266	5.6		*	
	014	0.299	892	3.9		0.297	4.0
	024	0.307	157	0.7			
	034	0.319	636	2.8		0.320	1.5
	044	0.336	24	0.1			
	104	0.339	322	1.4	2.9	0.340	2.5
	114	0.341	223	1.0			
	124	0.348	112	0.5			
	054	0.357	64	0.3	1.2	0.361	1.0
	134	0.359	100	0.4			
	144	0.375	114	0.5			
	064	0.381	2	0			

* Not observed due to fibre orientation.

DISCUSSION

(a) *Description of the structure*

The structure in its final form consists of two chains running in opposite directions with screw axes lying along the chain directions. There are four asymmetric N-acetylglucosamine residues per unit cell. The 1,4 type of bonding through an oxygen atom links the adjacent rings, which are "chair" shaped while the "planes" of the rings are perpendicular to the short repeat $a = 4.69$ Å. Fig. 6 represents the *a*-projection of the final structure, while Fig. 7 represents the *c*-projection with only half the unit cell along the fibre axis *c* projected. That is, it is a projection of just two asymmetric units which are approximately at the same height and belong to adjacent chains running in opposite directions. From Figs. 6 and 7 it can be seen that the plane of the amide group is almost parallel with the *ab*-plane, and is very slightly inclined to it.

A list of bond distances and associated bond angles are given in Table IV for one asymmetric unit of the structure. The bond distances are expected to be correct to about ± 0.05 Å, while the bond angles may not deviate by more than ± 3° from the listed values. The table also includes the bond distances and bond angles of the three hydrogen bonds present per single asymmetric unit of the structure.

(b) *Comparison with infra-red data*

It is interesting to note that the orientation of the amide group in the proposed structure is in good agreement with the infra-red absorption data published by Darmon and Rudall[2]. These workers found absorption frequencies indicating that the C=O and NH groups participate in intergroup hydrogen bonding of the type N–H . . . O=C, and that the strength of these hydrogen bonds were comparable with those in polyamides and proteins. They found that the N–H stretching band at 3265 cm^{-1} and the C=O stretching band at 1656 cm^{-1} both exhibit perpendicular dichroism, as also the N–H deformation band at 1560 cm^{-1}. Now the latter band can be shown to arise from the deformation of the N–H bond in the plane of the amide group, and it is thus apparent from the above data that the plane of the amide group as well as the C=O and N–H bonds themselves should be roughly perpendicular to the chain axis. The hydrogenic stretching absorption frequency has also probably overlapped with the N–H stretching frequency around 3250 cm^{-1} to give strong perpendicular dichroism in this vicinity.

The second feature of interest in the structure is the internal hydrogen bond O–H . . . O=C which, as can be seen from Figs. 6 and 7, is approximately parallel to the fibre axis. Darmon and Rudall[2] have observed an absorption band at 3485 cm^{-1} which has parallel dichroism. This band has been attributed by them to be a weakly bonded O–H vibration. This frequency or the one at 3445 cm^{-1} (the latter more likely) can thus be associated with the hydrogenic stretching frequency of a weakly bonded O–H . . . O=C hydrogen bond with parallel dichroism. The band at 2750 cm^{-1} may also be attributed to this bond. Thus the internal hydrogen bond is also in agreement with infra-red data.

The third feature of interest in the structure is the lateral O–H . . . O hydrogen bond between the hydroxymethyl groups of the shorter side-chains of main adjacent chains running in opposite directions. From the projections given in Fig. 9 and 10 it is clearly seen that this hydrogen bond is perpendicular to the fibre axis. Now

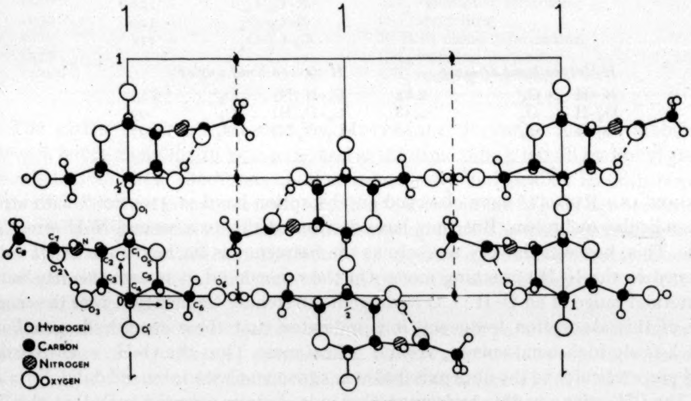

Fig. 6. The *a*-projection of the final structure.

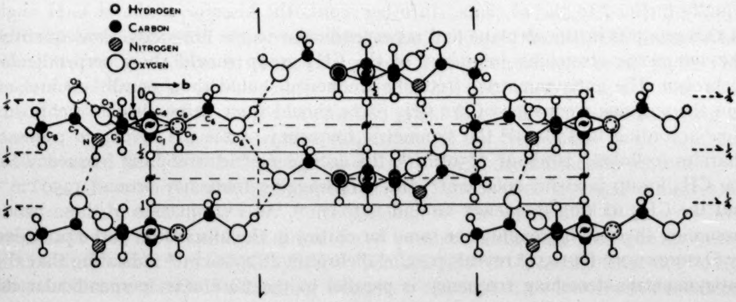

Fig. 7. The *c*-projection (fibre) of one half of the unit cell along the *c*-axis of the final structure.

TABLE IV

BOND DISTANCES AND BOND ANGLES

Bond	*Bond distance (Å)*	*Atoms in bond*	*Angle (degrees)*
C_1-C_2	1.48	$O_5-C_1-O_1$	112
C_2-C_3	1.54	$C_2-C_1-O_1$	112
C_3-C_4	1.48	$O_5-C_1-C_2$	112
C_4-C_5	1.47	$C_1-C_2-C_3$	112
C_5-O_5	1.50	$C_2-C_3-C_4$	112
C_5-C_6	1.58	$C_3-C_4-C_5$	112
C_3-O_3	1.30	$C_4-C_5-O_5$	111
C_6-O_6	1.50	$C_5-O_5-C_1$	116
O_5-C_1	1.47	$O_5-C_5-C_6$	109
O_1-C_1	1.48	$C_4-C_5-C_6$	109
C_2-N	1.42	$C_5-C_6-O_6$	118
$N-C_7$	1.47	$C_4-C_3-O_3$	109
C_7-C_8	1.54	$C_2-C_3-O_3$	112
$C_7=O_2$	1.10	C_3-C_2-N	107
		C_1-C_2-N	109
		C_2-N-C_7	111
		$N-C_7-C_8$	128
		$N-C_7=O_2$	120
		$C_8-C_7=O_2$	112
Hydrogen bond distance		*Hydrogen bond angles*	
$N-H \ldots O_2'$	2.82	$C_7-N(H) \ldots O_2'$	115
$O_3-H \ldots O_2$	2.58	$C_6-O_6(H) \ldots O_6'$	97
$O_6-H \ldots O_6'$	2.80	$C_3-O_3(H) \ldots O_2$	97

DARMON AND RUDALL[2] have observed an absorption band at 3100 cm^{-1} with strong perpendicular dichroism. But they have attributed this to a second N–H stretching mode. This, however, is very unlikely as the frequency is far below the usual values assigned to the N–H stretching mode. On the other hand, it is conveniently within the normal range of an O–H . . .O hydrogenic stretching frequency. From the magnitude of this absorption frequency it is indicated that these cross hydrogen bonds have a fairly high bond energy, around 7 kcal/mole. Thus the O–H . . . O hydrogen bond perpendicular to the fibre axis is also in agreement with infra-red data.

The CH_2 group in this hydroxymethyl side-chain is oriented such that the CH_2 plane is roughly parallel to the fibre axis (Figs. 6 and 7), with each of the C–H bonds equally inclined to the *ab* plane. In other words the bisector of the H–C–H angle of this group is in the *ab* plane and is perpendicular to the fibre axis. Consequently, the symmetric stretching frequency of the CH_2 group should show perpendicular dichroism. The antisymmetric stretching frequency should show parallel dichroism, and the wagging frequency of the CH_2 plane should show perpendicular dichroism. Now according to TSUBOI[10] the symmetric frequency of the CH_2 group in polysaccharides (cellulose) is about 2850 cm^{-1}, the antisymmetric stretching frequency for the CH_2 group is about 2960 cm^{-1}, the CH_2 wagging frequency around 1250 cm^{-1} and the CH_2 rocking frequency around 1030 cm^{-1}. An examination of these bands (assuming them to be roughly the same for chitin) in the absorption curve provided by DARMON AND RUDALL[2] reveals parallel dichroism at 2960 cm^{-1} indicating that the antisymmetric stretching frequency is parallel to the fibre axis; perpendicular dichroism at 2840 cm^{-1} indicating that the symmetric stretching frequency of the

CH_2 group is perpendicular to the fibre axis; and perpendicular dichroism at 1270 cm^{-1} indicating that the wagging frequency of the CH_2 plane is perpendicular to the fibre axis. Also strong parallel dichroism at 1030 cm^{-1} in the spectrum indicates that the in-plane rocking frequency of the CH_2 plane is parallel to the fibre axis. This very close agreement of the infra-red absorption data for the various modes of vibration of the CH_2 group thus confirms the orientation of the hydroxymethyl side-group containing the CH_2 group. A complete list of infra-red absorption data is given in Table V.

TABLE V

INFRA-RED ABSORPTION DATA ACCORDING TO DARMON AND RUDALL

Absorption frequency (cm^{-1})	*Observed dichroism*	*Dichroism expected from present structure*	*Assignment*
3485	//	//	O–H stretching
3445	//	//	O–H . . . O=C hydrogenic stretching
3265	⊥	⊥	N–H stretching
3100	⊥	⊥	O–H . . . O hydrogenic stretching
2960	//	//	CH_2 antisymmetric stretching
2840	⊥	⊥	CH_2 symmetric stretching
1656	⊥	⊥	C=O stretching
1560	⊥	⊥	N–H in plane deformation
1270	⊥	⊥	CH_2 wagging frequency
1030	//	//	CH_2 rocking frequency

The earlier structure proposed by MEYER AND PANKOW[1] had no stabilizing hydrogen bonds along the 19.25 Å axis, and at the same time it left all hydroxyl groups unbonded. Both these conditions are stereochemically undesirable. In both respects the present structure is more satisfactory. Further it is in fairly good agreement both with x-ray diffraction data and infra-red absorption data. The most interesting aspect of this structure determination is the fact that the configuration which from the accepted criteria of stereochemistry may be said to be the most satisfactory, is the one which is actually taken up.

The results of this investigation confirm the unit cell parameters and the orthorhombic space group $P2_12_12_1$ proposed by CARLSTRÖM[11]. However, they have led to a straight chain structure, instead of the buckled structure adopted by CARLSTRÖM, and also to somewhat different types of hydrogen bonds. We feel that the present structure should be considered to be more satisfactory for the following reasons.

In the structure proposed by CARLSTRÖM the chains are buckled, with the result that the hydroxymethyl side-groups cannot be hydrogen bonded, the distance between the oxygen atoms of these side groups in adjacent chains being 3.3 Å. This renders the structure unstable since the chains are unbonded along the long 19 Å axis. On the other hand, in the attempt to make this hydrogen bond as strong as possible, we found that the relative disposition of the two chains in the unit cell thus obtained also gave the best agreement with the X-ray diffraction pattern.

Now the infra-red absorption spectrum of DARMON AND RUDALL[2] indicates no absorption band at 3640 cm^{-1} corresponding to the unbonded O–H stretching frequency, but only bands at lower wave numbers indicating that the hydroxyl groups in the structure are all hydrogen bonded. Now if we assume that due to hydrogen bonding the O–H stretching frequency has shifted to lower wave numbers, and has overlapped either with the true N–H stretching frequency observed at

3265 cm^{-1} or with its strongly dichroic companion at 3100 cm^{-1}, both of which show perpendicular dichroism, then lateral hydrogen bonding between adjacent chains becomes essential, the hydrogen bonds being perpendicular to the fibre axis.

Now the reason for the buckling of the chains in CARLSTRÖM's structure seems to be three-fold. In the first place, bonding is achieved between the short hydroxyl group of one asymmetric residue and the ring oxygen of the next. If this bond really exists, it must be very strong to force the chains to buckle. But the small frequency shift from 3640 cm^{-1} to the observed band at 3485 cm^{-1} indicates that the hydrogen bonding is fairly weak. Furthermore, the fact that the ring oxygen is almost saturated and is not sufficiently negative indicates that this hydrogen bond if present would be rather weak. This is also confirmed by the fact that in the structure of sucrose by BEEVERS, MCDONALD *et al.*[12], the ring oxygen does not take part in hydrogen bonding, while in the structure of α-glucose by MCDONALD AND BEEVERS[13] the ring oxygen only bonds weakly with the hydrogen atom attached to the oxygen atom O_1. On the other hand the hydroxyl group can be conveniently hydrogen bonded to the oxygen of the amide group in the same asymmetric unit which is sufficiently negative to take part in double hydrogen bonding. This bonding is also parallel to the fibre axis and is rather weak, and thus accounts for the absorption band at 3485 cm^{-1} having parallel dichroism.

The second reason for buckling of the chains is the short contact between the nitrogen atom of one residue and the carbon atom C'_6 of the residue above. Now in the present straight chain structure, this interatomic distance is 3.1 Å, which is really not a short contact since a number of instances of such short interatomic distances between C and N atoms are known. This distance can in fact be increased to 3.4 Å by making slight alterations in the interatomic angles and thereby slightly changing the atomic coordinates of the various atoms in the side chain. This, however, calls for refinement of the structure which is reserved for further work.

Thirdly, buckling of chains was resorted to in order to remove the steric interference between the hydrogen atoms on the carbon atoms C_1 and C'_4. In the present straight chain structure the distance between these hydrogen atoms (see Fig. 6) is about 2.3 Å. A distance larger than 2.0 Å between the hydrogens seems to be generally permissible. In fact a van der Waals contact distance of 2.3 Å between the hydrogens of CH_2 groups occurs in the structure of diglycine hydrochloride (HAHN AND BUERGER[14]). Thus, no serious steric interference could be considered to exist in the straight chain configuration proposed in this paper.

ACKNOWLEDGEMENT

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REFERENCES

[1] K. H. MEYER AND G. W. PANKOW, *Helv. Chim. Acta*, 18 (1935) 589.
[2] S. E. DARMON AND K. M. RUDALL, *Discussions Faraday Soc.*, 9 (1950) 251.
[3] K. H. MEYER AND H. MARK, *Ber.*, 61 (1928) 1936.
[4] K. H. MEYER AND H. WEHRLI, *Helv. Chim. Acta*, 20 (1937) 353.

[5] M. BERGMANN, L. ZERVAS AND E. SILBERWEIT, *Naturwissenschaften*, 19 (1931) 20.
[6] K. M. RUDALL, *Progr. Biophys. and Biophys. Chem.*, 1 (1950) 39.
[7] S. E. DARMON AND G. B. B. M. SUTHERLAND, *Nature*, 164 (1949) 440.
[8] E. G. COX AND C. A. JEFFREY, *Nature*, 143 (1939) 894.
[9] H. VIERVOLL AND O. ÖGRIM, *Acta Cryst.*, 2 (1949) 277.
[10] M. TSUBOI, *J. Polymer Sci.*, 25 (1957) 159.
[11] D. CARLSTRÖM, *J. Biophys. Biochem. Cytol.*, 3 (1957) 669.
[12] C. A. BEEVERS, T. R. R. MCDONALD, J. H. ROBERTSON AND F. STERN, *Acta Cryst.*, 5 (1952) 689.
[13] T. R. R. MCDONALD AND C. A. BEEVERS, *Acta Cryst.*, 5 (1952) 654.
[14] T. HAHN AND M. J. BUERGER, *Z. Krist.*, 108 (1957) 419.

STUDIES ON PREPARATION OF BACTERIAL CELL WALLS AND CRITERIA OF HOMOGENEITY

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SUMMARY

Group A streptococci can be disrupted in a sonic vibrator in the presence of glass beads to yield large cell wall fragments. A technique is described, involving sucrose zone centrifugation, for collecting cell walls with a high degree of purity, and in good yield, suitable for studies on biological properties. It is concluded that cell walls prepared by the technique of sucrose zone centrifugation display a relatively high degree of homogeneity as indicated by electron microscopy and moving boundary electrophoresis. Immunological studies show the cell wall preparations to be antigenically complex although not necessarily contaminated with non-cell wall antigens.

INTRODUCTION

Recent studies have been reported on the toxic properties of cellular components of group A streptococci[1,2]. Among these components is a factor which produces a chronic, remittent and intermittent multinodular lesion of dermal connective tissue following its intradermal injection into rabbits[1]. This toxic material has been shown to be a complex macromolecule containing the group-specific C polysaccharide[3,4], which is a major constituent of the group A streptococcus cell wall. Subsequent studies have been reported on the relationship between toxic activity and the macromolecular properties of size and configuration of cell wall fragments[5,6]. To pursue these studies further, investigations have been conducted on the efficient preparation of a homogeneous suspension of bacterial cell walls in relatively large quantities.

The method commonly employed for the purification of bacterial cell walls was introduced by Dawson[7], and extended by Salton and Horne[8]. This technique involves differential centrifugation of disrupted cells and washing sediments with buffers. There have been few modifications of this basic technique, other than treatment with ribonuclease and proteolytic enzymes as employed, for example, by Cummins and Harris[9]. Extensive studies on the composition of cell walls derived from group A streptococci have been carried out by McCarty[10], and Barkulis *et al.*[11], using the technique of differential centrifugation.

The present communication describes a modification of the usual technique for preparation of bacterial cell walls, which involves sucrose zone centrifugation. In addition to electron microscopy, some electrophoretic and immunologic studies on these purified preparations are reported.

METHODS AND RESULTS

Growth of the organisms

A group A, type 1, streptococcus was grown in Todd-Hewitt broth prepared from fresh beef heart[1]. Prior to inoculation, a glucose–phosphate buffer was added to give a glucose concentration of 0.3 %. The medium was inoculated with 4 ml/l of a starter culture in the late logarithmic phase of growth. The culture was incubated for 16–18 h at 35°. Cells were harvested in a Servall S-2 centrifuge, washed twice in cold saline and once with distilled water.

Rupture of the cells and collection of the cell walls

Low percentage rupture of cells by grinding techniques and the high degree of fragmentation and solubilization of cell walls occurring on disruption with sonic vibration, prompted attempts to combine the principles into a single procedure utilizing available equipment. Incorporation of fine glass beads into the vibration mixture was found to produce high percentage rupture. Values obtained for solubilized rhamnose indicated that the mechanism of rupture differs appreciably from sonic vibration alone[12]. The time of vibration was adjusted to obtain maximum rupture with minimum fragmentation. The washed cells from 2 l of culture, suspended in water to a total volume of 10 ml, were mixed with 10 ml Flexolite* No. 18 glass beads. This mixture was vibrated for 25 min at maximum amplitude in a Raytheon 9 kc sonic oscillator. The temperature was maintained at 4° by circulating ice water through the cooling system of the treatment unit.

The vibrated material was transferred to a conical centrifuge tube. Beads and cell material were separated by allowing the beads to settle, withdrawing the supernate, and washing the beads with small aliquots of water which were added to the supernate. This suspension was centrifuged for 30 min at 10,000 rev./min in a Spinco No. 30 rotor, and the supernate was discarded. A rough separation of cell walls and cells was visible in the layered sediment. The more loosely packed cell wall layer was rinsed from the lower cell layer. Two additional sedimentations of both fractions, followed by segregation of the layers, left a pellet composed almost entirely of whole cells. The whole cells thus recovered were combined and utilized again to improve the yield. The suspension from the upper layer of sediments contained mostly cell walls with "granules" and few whole cells.

Sedimentation through sucrose

A sucrose gradient was prepared by using a large bore pipette to layer solutions of decreasing sucrose densities in a 40-ml glass centrifuge bottle. Bottles were layered from the bottom with solutions having the following densities: 1.30 (1.5 ml); 1.25 (4 ml); 1.20 (4 ml); 1.15 (4 ml); 1.10 (4 ml); 1.05 (4 ml); and 1.025 (4 ml). These were placed in the cold for 16 h to allow gradient formation by diffusion.

A uniform dispersion of the cell wall suspension was obtained by placing it in a cellulose centrifuge tube and subjecting it to 30 sec sonic vibration in the cup of the

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* Flexolite Mfg. Corp., St. Louis, Mo.

Raytheon oscillator containing 15 ml of water. 3 ml of this suspension was then layered over the sucrose gradient and the bottle tightly stoppered. The bottles were centrifuged at 2,700 rev./min in an International refrigerated centrifuge with a No. 269 rotor, for 1.75 h with controlled acceleration and deceleration according to the method of ANDERSON[13]. To produce uniform acceleration, the auto-transformer speed control was advanced to the setting required to turn the rotor. When the speed reached 100 rev./min, the speed control was advanced 1 unit. After 10 sec, the speed control was advanced an additional unit each 5 sec until the tachometer reached 2,700 rev./min. The speed control was turned back to maintain the speed. Deceleration was accomplished by turning back the speed control 1 unit each 5 sec to a setting which just kept the rotor from stopping.

Three distinct bands were visible and could be removed separately with a pipette. Samples from each band were examined for the presence of whole cells by phase microscopy and Gram's stain. Most of the cells were confined to the lowest band and were discarded. Those bands with no whole cells were combined. The top layer, composed largely of "granules", could be removed at this point. However, since an appreciable quantity of cell wall was present in this layer, segregation of these elements was postponed until after papain treatment, at which time complete separation is more readily achieved. Lower levels of the middle band with rare cells were centrifuged separately. The fractions were washed with water by sedimenting at 17,500 rev./min for 30 min in a Spinco No. 30 rotor. The loose upper layers were again rinsed from the sediments containing the few remaining whole cells and were combined with the cell-free layers. Fractions were resuspended and washed twice with water, and 3 times with pH 7, $I = 0.1$, phosphate buffer by sedimenting at 10,000 rev./min for 20 min in the Spinco No. 30 rotor. The latter washing has been shown to remove ribonucleic acid[14].

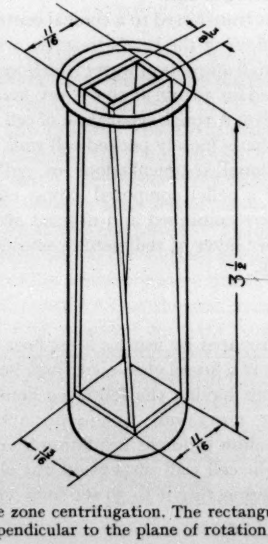

Fig. 1. Sector cell for sucrose zone centrifugation. The rectangular surface lies along the radii of the rotor and perpendicular to the plane of rotation. Dimensions in inches.

The bottles used for the gradient separation in this report, though workable and practicable from the point of view of sterilization and availability, are not ideal. Since the sides of the bottle are parallel, during centrifugation a percentage of particles fanning out from the center of rotation strike the sides, pile up, and slide down the sides. Sharper separation has been achieved by substituting sector cells, constructed to eliminate wall effects. Fig. 1 is a diagrammatic representation of a sector cell constructed for use in sucrose zone centrifugation. The capacity is approx. 17 ml. The cells, constructed from lucite, were held in place in cellulose centrifuge tubes (Lusteroid, IEC, 50 ml), by embedding in a low shrinkage casting plastic*. The cellulose tube and the hardened plastic also provide adequate support for the cell during centrifugation. The body of the cell was constructed from 1/16 inch lucite sheet, the joints fused with acetone. Two rectangular surfaces of the cell were arranged perpendicular to the plane of rotation and lying along radii of the rotor. The other two surfaces were fixed parallel to each other and were trapezoidal. The base dimensions were limited by the internal diameter of the cellulose centrifuge tube.

Papain treatment

Papain was chosen to remove proteins associated with the cell wall because of its wide range of specificity. This enzyme removes 45–50 % of the nitrogen, and is more effective than pepsin, trypsin or chymotrypsin[3]. The cell walls were resuspended in 8.9 ml of water and placed in a small flask. 1 ml pH 7, $I = 0.1$, phosphate buffer was added. 2 mg reduced papain (Nutritional Biochemicals Corporation) in 0.1 ml was added to the suspension, and the pH was adjusted to 7.0. Two drops of chloroform were added, and the flask was incubated with occasional mixing for 5 h at 37°. After incubation, the material was centrifuged for 20 min at 17,500 rev./min (Spinco No. 30 rotor). The sediment was resuspended in water by subjecting it to 30 sec sonic vibration, and sucrose sedimentation was repeated as before. The dense band nearest the top of the gradient, containing many granules was removed and the broad middle band collected. The bands were washed three times with water. The sediment containing the "granules" appeared viscous and transparent. The cell walls appeared opaque and white.

Analysis of fractions: chemical

In order to evaluate the procedure, aliquots for analysis were taken at different stages in the purification process. Rhamnose values, obtained by the method of DISCHE AND SHETTLES[13], were used as an indication of cell wall material in the different fractions, since this sugar is confined to the cell wall structure[10]. Nitrogen values, determined by a micro-Kjeldahl method utilizing Nessler's reagent, were used to calculate rhamnose to nitrogen ratios, which provide an index of relative purity. Table I gives the results of a sample extraction of cell walls from the cells harvested from 5 l of culture medium. The rhamnose of the final preparation indicates that 35 % of the cell walls were recovered by this procedure. The largest loss occurred prior to sucrose gradient separation. Relatively little solubilization of the cell wall occurred during sonic vibration with glass beads, as indicated by the small percentage of rhamnose found in the supernates following centrifugation at 10,000 rev./min for

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* Kosto Hobby Crafts Plastic Corp., Washington, D.C. (U.S.A.).

TABLE I

COMPOSITION OF FRACTIONS FROM PURIFICATION PROCEDURE

	Percent of original		*Ratio of Rhamnose*
	Nitrogen	*Rhamnose*	*Nitrogen*
Whole cells	100	100	0.66
Supernates of vibration*			
First vibration	36.9	7.3	0.129
Second vibration**	17.4	3.8	0.143
Total	54.4	11.1	0.134
Cell walls (pre-sucrose)	17.5	64.1	2.22
Cell walls (pre-papain)	13.6	55.3	2.66
"Granules"	2.2	9.9	2.94
Cell walls	4.5	35.1	5.07

* Supernates from centrifugation at 10,000 rev./min, for 30 min.

** From revibration of recovered cells.

30 min (11,500 × g). By comparison, a cell preparation sonic vibrated for 1 h without beads requires centrifugation for 1 h at 144,000 × g to sediment a comparable percentage of rhamnose. On the other hand, 72 % as much nitrogen is solubilized by sonic vibration with glass beads as that solubilized by sonic vibration alone.

The u.v. absorption spectrum of sonic vibrated cell walls was measured in a Beckman model DU spectrometer. Cell walls which had been treated with papain and washed 3 times with pH 7. phosphate buffer were suspended in water and subjected to sonic vibration without glass beads for 3 h and centrifuged at 10,000 rev./min for 30 min. The resulting supernatant was used for absorption measurements. The curve showed essentially only terminal absorption in the far u.v., with no peak absorption in the region of either 280 mμ or 260 mμ.

Electron microscopy

Fig. 2 shows a composite of electron micrographs representing different phases of purification. Preparations on collodion film were shadowed with chromium at an angle of 15°. Frame 3 shows a mixture of cell walls and "granules". Frames 1 and 2 indicate the high degree of separation achieved between the cell walls and the cellular "granules", and illustrate also the failure of papain to change the gross morphology of these elements.

The occurrence of similar "granules" following disruption of streptococcal cells has been noted by others[8, 12]. The nature and origin of these structures is uncertain. SLADE *et al.*[12] report that the mass of the "granules" appears to increase as sonic vibration is continued up to 4 h. Some characteristics of these "granules" can be concluded from the data presented here since, as shown in Fig. 2, the "granule" structures have been separated from nearly all recognizable cell wall material. This preparation has a relatively high rhamnose content, is resistant to papain digestion, and does not show any peak absorption at 260 or 280 mμ. Thus the "granules" appear to be related to the cell wall structure although chemically and morphologically distinguishable. At the present time it seems likely that they represent a complex between certain cell wall fragments and other cell components which aggregate during exposure to sonic vibration.

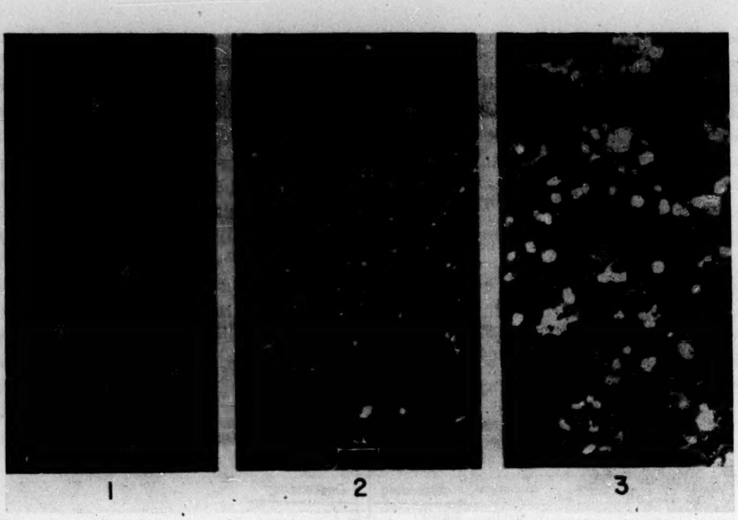

Fig. 2. Composite of electron micrographs showing phases of purification. Shadowed with chromium at an angle of 15°. Frame 1, purified, papain-treated cell wall suspension. Frame 2, "granules" from upper layer of sucrose zone centrifugation. Frame 3, mixture of cell walls and "granules" prior to sucrose zone centrifugation.

No whole cells were found by examining samples of the cell wall preparation by electron microscopy or by phase microscopy. A loopful failed to give evidence of viable cells on blood-agar medium.

Electrophoretic analysis

As a further investigation on the properties of the purified cell wall preparation, electrophoretic analysis was conducted on a suspension dispersed by sonic vibration. Initially, electrophoresis of whole cell walls was attempted. Although there was no evidence of heterogeneity, turbidity was too great for a critical observation of the complete boundary. Therefore, an aliquot of cell wall suspension containing approx. 50 mg dry wt. was suspended in 15 ml veronal buffer, pH 8.6, $I = 0.1$, and sonic vibrated for 2.5 h without glass beads. The suspension was dialyzed for 18 h against buffer, centrifuged for 15 min at 2000 rev./min in an International refrigerated centrifuge (rotor No. 269), and analyzed by moving boundary electrophoresis in a Spinco model H electrophoresis instrument. Fig. 3 shows the descending and ascending patterns obtained at various intervals with a potential gradient of 3.49 V/cm. Considering the complexity of a crude cell extract[12], the pattern shows remarkable homogeneity. There is a faster moving component representing 6 % of the total area, resolvable in both boundaries. Resolution of the principal, skewed boundary into separate components is not observed after 300 min under these conditions of pH, etc. The mobility of the principal peak is $-5.9 \cdot 10^{-5}$ cm^2/V/sec, measuring ∂x to the peak of the boundary. Practical enantiography exists between descending and ascending boundaries throughout the course of current passage. The skewness of the curve could reflect a polymerization reaction of the type $n\mathrm{B} \rightarrow \mathrm{B}n$, the B monomers being produced during vibration. If such a form of modality exists the skewed peak would be considered a single boundary since a homogeneous phase was not produced between the 2 maxima[16]. It is noteworthy that there is enantiographic skewness of the ascending boundary. This is difficult to explain by either the theory of polymerization and dissociation, or as a bimolecular association[16]. Observations noted during the study of biological activity of vibrated cell walls which suggest a tendency of cell wall fragments to polymerize or to interact include: (a) the rapid disappearance, on standing, of a hemolysin from vibrated cell walls[2]; and (b) the rapid increase in O.D. after initial reduction following treatment of certain cell wall fractions with *Streptomyces albus* enzyme[5].

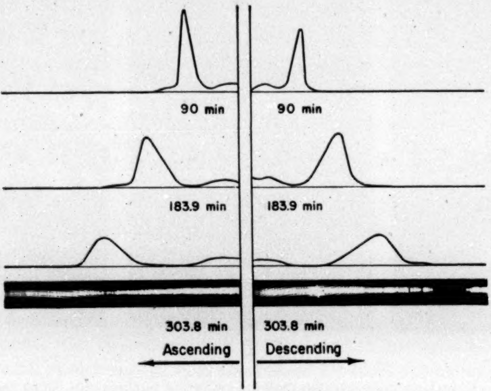

Fig. 3. Moving boundary electrophoretic patterns of cell wall suspension dispersed by 2.5 h sonic vibration. Veronal buffer pH 8.6, I 0.1, at a potential gradient of 3.49 V/cm. The boundary patterns at 90 min were photographed at different diaphragm slit angles.

Immunological studies

Agar diffusion experiments were designed to estimate the immunological complexity of the cell wall preparation. Modifications of both the OAKLEY[17], and the OUCHTERLONY[18] methods were employed. The antisera used were prepared in rabbits against killed streptococcal cell vaccine and against purified streptococcal cell walls[4]. A portion of the antiserum against whole cells was absorbed with a sonic extract of a group A streptococcal variant which produces no group-specific C polysaccharide[19]. This yielded an antiserum which had antibodies reacting only with C polysaccharide[4]. The variant strain, B-455, was kindly supplied by Dr. R. C. LANCEFIELD. These antisera were arranged in agar in positions opposing the sonic vibrated cell wall suspension, and three other streptococcal preparations: the group-specific C polysaccharide obtained from cell walls by FULLER's formamide technique[20]; a sonic extract of whole cells; and a fraction of the whole cell extract (75p60-4), which contained high rhamnose content and an ultraviolet absorption spectrum similar to vibrated cell walls[3]. Table II summarizes the reactions noted with these systems.

The major precipitin band between each antiserum and the vibrated cell wall preparation was common to the band obtained with the isolated C polysaccharide. The presence of at least two additional antigens in the cell wall suspension is established by the reactions between the vibrated cell walls and the antisera against the cell wall preparation and against the whole cells. The presence of another antigen is implied by a fourth precipitin band occurring between the partially purified extract, 75p60-4, and antiserum prepared against the purified cell wall preparation. The absence of this band in reactions with vibrated cell walls suggests that this antigen is present in a quantity too small to produce a visible precipitin reaction in these systems. With all antigen preparations which have been studied the absorbed antiserum shows a single precipitin band, except when reacting with cell wall preparation, as noted in Table II. It is unlikely that a contaminating antigen which was not present in crude cell extracts, would be detectable in the purified cell walls. The possibility is being investigated that the two precipitin lines observed here represent the same antigenic group occurring in fragments with sufficiently different physical properties to produce distinct lines.

TABLE II

PRECIPITIN REACTIONS OF STREPTOCOCCAL FRACTIONS IN AGAR

	Antigen preparations			
	C poly-saccharide	*Vibrated cell wall*	*Whole cell extract*	*75p60-4*
Antiserum to whole cells	1*	3	5	4
Absorbed antiserum**	1	2	1	1
Antiserum to cell wall	1	3	3	4

* Number of precipitin bands developing in agar between antigen and antiserum.

** Antiserum to whole cell vaccine absorbed with a sonic extract of a group A variant producing no group-specific C polysaccharide.

DISCUSSION

The primary purpose of obtaining purified cell wall suspensions was to pursue studies on toxic properties which are derived from the cell wall[5,6]. When applied to disrupted Group A streptococci, methods relying on differential centrifugation alone do not yield a product possessing the purity required for such investigations. These methods were also found to be inadequate for removal of whole cells without large loss of cell walls. Therefore, the modification involving sucrose gradients was introduced. The use of sucrose zone centrifugation serves a dual function. Sucrose, by absorption into the cells increases the apparent density of the cells, thereby increasing the sedimentation differential between them and the cell walls. Compared with conventional centrifugation, the effective distance of sedimentation is also increased, thus increasing and maintaining the separation between elements with different sedimentation rates.

Electron microscopy has often been used to estimate gross particulate impurities in cell wall preparations and the preparations reported here are very homogeneous by this criterion. However, this technique is not satisfactory for studies attempting to assign a biological property specifically to the cell wall structure. Most studies on cell walls have made no attempt to assess the presence of non-particulate contaminating components which, biologically, could be very significant.

Although electrophoretic analysis has been reported on the group specific polysaccharides of streptococci prepared by LANCEFIELD technique[21], and on extracts of streptococci solubilized by sonic vibration or shaking with glass beads[22], electrophoresis has not been used to estimate heterogeneity of cell wall preparations. Evidence presented here illustrates the feasibility of reducing the turbidity of the solution by sonic vibration of the cell wall suspension, so that resolution of boundaries by either the cylindrical lens or Rayleigh fringe optical techniques is possible. Dispersion by sonic oscillation apparently does not affect the mobility or electrophoretic heterogeneity of the preparation. However, the degree of polydispersity of the suspension treated in this manner has not been determined.

The immunological analysis of the purified cell walls provides information on the antigenic complexity of the cell wall preparation. Reactions obtained between vibrated cell walls and antisera prepared against the cell wall preparation established the presence of at least two antigenic moities other than the group-specific C polysaccharide. Whether these antigens are an integral part of the cell wall or reflect a degree of contamination with non-cell wall material cannot be ascertained from these experiments. A study comparing protoplasts and protoplast membrane antigens with the cell wall preparations should clarify this question.

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REFERENCES

[1] J. H. SCHWAB AND W. J. CROMARTIE, *J. Bacteriol.*, 74 (1957) 673.
[2] E. A. SHARPLESS AND J. H. SCHWAB, *J. Bacteriol.*, 79 (1960) 496.
[3] J. H. SCHWAB, W. J. CROMARTIE AND B. S. ROBERSON, *J. Exptl. Med.*, 109 (1959) 43.
[4] J. H. SCHWAB AND W. J. CROMARTIE, *J. Exptl. Med.*, 111 (1960) 295.
[5] B. S. ROBERSON, J. H. SCHWAB AND W. J. CROMARTIE, *Bacteriol. Proc.*, (1959) 67.
[6] B. S. ROBERSON AND J. H. SCHWAB, to be published.
[7] I. M. DAWSON, in A. A. MILES AND N. W. PIRIE, *The Nature of the Bacterial Surface*, Blackwell Scientific Publications Lim. Oxford, 1949, p. 119.
[8] M. R. J. SALTON AND R. W. HORNE, *Biochim. Biophys. Acta*, 7 (1951) 177.
[9] C. S. CUMMINS AND H. HARRIS, *J. Gen. Microbiol.*, 14 (1956) 583.
[10] M. MCCARTY, *J. Exptl. Med.*, 96 (1952) 569.
[11] B. S. TEPPER, J. A. HAYASHI AND S. S. BARKULIS, *J. Bacteriol.*, 79 (1960) 33.
[12] H. D. SLADE AND J. K. VETTER, *J. Bacteriol.*, 72 (1956) 27.
[13] N. G. ANDERSON, in G. OSTER AND A. W. POLLISTER, *Physical Techniques in Biological Research*, Vol. 3, Academic Press, New York, 1956, p. 299.
[14] S. S. BARKULIS AND M. F. JONES, *J. Bacteriol.*, 74 (1957) 207.
[15] Z. DISCHE AND L. B. SHETTLES, *J. Biol. Chem.*, 175 (1948) 595.
[16] I. G. LONGSWORTH, in M. BIER, *Electrophoresis Theory, Methods and Applications*, Academic Press, New York, 1959, p. 91.
[17] H. R. G. DEICHER, H. R. HOLMAN AND H. G. KUNKEL, *J. Exptl. Med.*, 101 (1959) 97.
[18] S. P. HALBERT, L. SWICK AND C. SONN, *J. Exptl. Med.*, 101 (1955) 557.
[19] M. MCCARTY AND R. C. LANCEFIELD, *J. Exptl. Med.*, 102 (1955) 11.
[20] A. T. FULLER, *Brit. J. Exptl. Pathol.*, 19 (1938) 130.
[21] N. J. BERRIDGE AND C. A. E. BRIGGS, *Nature*, 174 (1954) 486.
[22] E. L. HESS AND H. D. SLADE, *Biochim. Biophys. Acta*, 16 (1955) 346.

BIOPHYSICAL PROPERTIES OF BACTERIOPHAGE T2

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SUMMARY

Biophysical properties of bacteriophage T2 and T2 "ghosts" have been studied using the ultracentrifuge, the electron microscope and the diffusion apparatus. The molecular weight of T2 at either pH 7 or pH 5 has been found to be $215 \cdot 10^6$. The protein portion of the head of the intact particles or of the DNA-free phage ghost has been found to exist in two forms, depending on both pH and temperature. One head form of intact T2 is approximately 15% longer than the other; 119 mμ by 80 mμ *vs.* 103 × 80 mμ. Raising either the temperature or the pH favors the formation of the longer head form. This change in head form accounts for the dual sedimentation phenomenon observed now for T2 ghosts and reported previously for intact T2. Estimates have been made of the thermodynamic changes involved in the transition of one form to the other.

INTRODUCTION

HOOK, BEARD, TAYLOR, SHARP AND BEARD reported in 1946 that T2 bacteriophage particles exhibit the unusual property of having two different sedimentation coefficients depending upon the suspending medium[1,2]. The sedimentation coefficient was about 700 S** in 0.9% NaCl and about 1000 S in 0.023 *M* $CaCl_2$. The appearance of these two forms was also dependent on the pH of the solvent, at a pH greater than 5.8, the 700 S (slow) form existed, while below pH 5.8, only the 1000 S (fast form) was observed. BENDET, SWABY AND LAUFFER[3] later demonstrated that the transition of the fast to the slow form took place in less than 2 min.

Two explanations for this dual sedimentation behavior of T2 have been proposed. SHARP *et al.*[2] suggested that orientation of specifically aggregated particles could result in two such sedimentation coefficients. However, PUTNAM presented evidence indicating that the particles were not oriented during sedimentation[4]. This conclusion was challenged[3] on the grounds that the particles could have been oriented already at field strengths lower than PUTNAM employed. It was argued that orientation of

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Abbreviations: PTA, phosphotungstic acid, DNA, deoxyribonucleic acid.

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** All sedimentation coefficients will be given in Svedberg units (10^{-13} sec) (S) and will be corrected, when possible, to a water basis at 20°. The sedimentation coefficient in pH 7 buffer will sometimes be referred to as *S7* and in pH 5 buffer as *S5*. Similar notations will be used for the diffusion coefficients at these pH values.

these unusually shaped viruses could be brought about by the density heterogeneity due to the DNA inside the viral head. Later TAYLOR, EPSTEIN AND LAUFFER[5] and BENDET *et al.*[3] measured the diffusion coefficients of T2 in pH 5 and pH 7 buffers, calculated the molecular weights of the two forms, and concluded that since both forms have similar molecular weights, the dual sedimentation phenomenon could not be due to aggregation. It seemed more likely that the difference in sedimentation properties was due to a change in the individual particles. BENDET *et al.*[3,6] then postulated that a reversible change in the shape of the phage particle occurred at the tip of the tail. Tail fibers were thought to be unwound or extended in the slow form thus retarding sedimentation while in the fast form the tail fibers were thought to be tightly wound and not to affect sedimentation. These views were based on what were considered discrepancies in the ratios of the diffusion coefficients to the sedimentation coefficients of the two forms and on the appearance of the virus in electron micrographs.

We have reexamined the diffusion properties of T2 phage and found no discrepancy between the sedimentation and diffusion constants. In view of the fact that the head of this particle contains over 90 % of the mass of the particle, it seemed possible that a change in the physical properties, such as a 42 % change in the sedimentation coefficient, would involve a change in the head structure. We have now found that the two sedimenting forms have different head lengths; the slower sedimentation form has a head length 13–16 mμ longer than the faster sedimenting form. It has also been found that the interconversion of the two forms is markedly affected by temperature.

EXPERIMENTAL

Materials

Stocks of T2r$^+$ were grown on *E. coli* B in liquid culture and purified and assayed by standard procedures[7]. Protein coats or "ghosts" of T2r$^+$ were prepared and purified by the osmotic shock method described by HERRIOTT AND BARLOW[8]. Whole phage and "ghosts" were stored in the cold in 0.9 % NaCl, containing 0.001 *M* $MgSO_4$. Under the proper conditions, these preparations all gave single peaks in the ultracentrifuge. The phosphate content of samples of these preparations was found to be 3.9 % by weight. This agrees well with the values of COHEN AND ANDERSON[9] for T2, SCHLESINGER[10] for WLL (presumably T2) and KOZLOFF AND PUTNAM[11] for T6, but is lower than the 5.0 to 5.2 % reported by TAYLOR[12] and HERRIOTT AND BARLOW[13] for T2.

Diffusion rates

The diffusion coefficients of the fast and slow forms of whole T2 were measured in the Spinco Model H apparatus taking into consideration the effects of both pH and temperature. Since, as will be shown later, lowering the temperature of the suspending buffer tends to convert the slow form into the fast form, the pH of the buffer used in measuring the diffusion coefficient of the slower form must be high enough so that only the slow form exists at the relatively low temperature of the diffusion bath. Whole T2 at a concentration of $3 \cdot 10^{12}$/ml was suspended in sodium phosphate buffer, ionic strength 0.1, at a pH of 5.78 or 7.85 and the bath was kept at 7.1°. The diffusion coefficients were measured by the Rayleigh fringe method[14] and corrected to a water

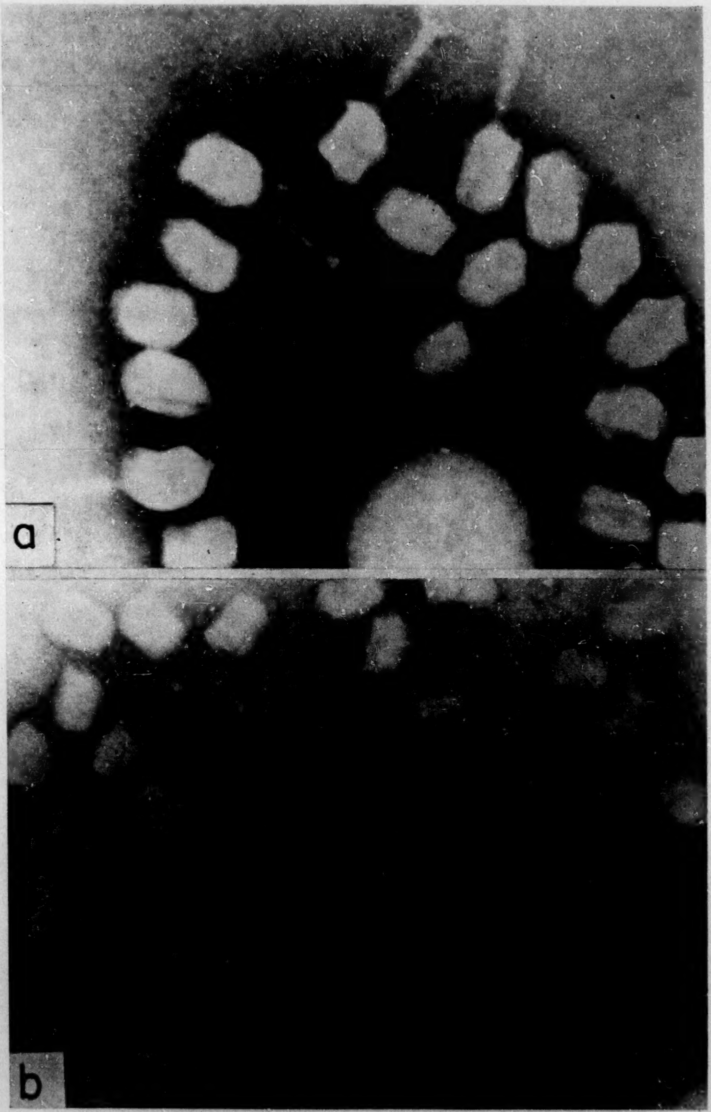

Fig. 1. Electron micrographs of the two forms of bacteriophage T2 using the PTA procedure after formol fixation. The magnification is 125,000 × for both pictures. (a) T2 at pH 7.0 and (b) T2 at pH 5.7.

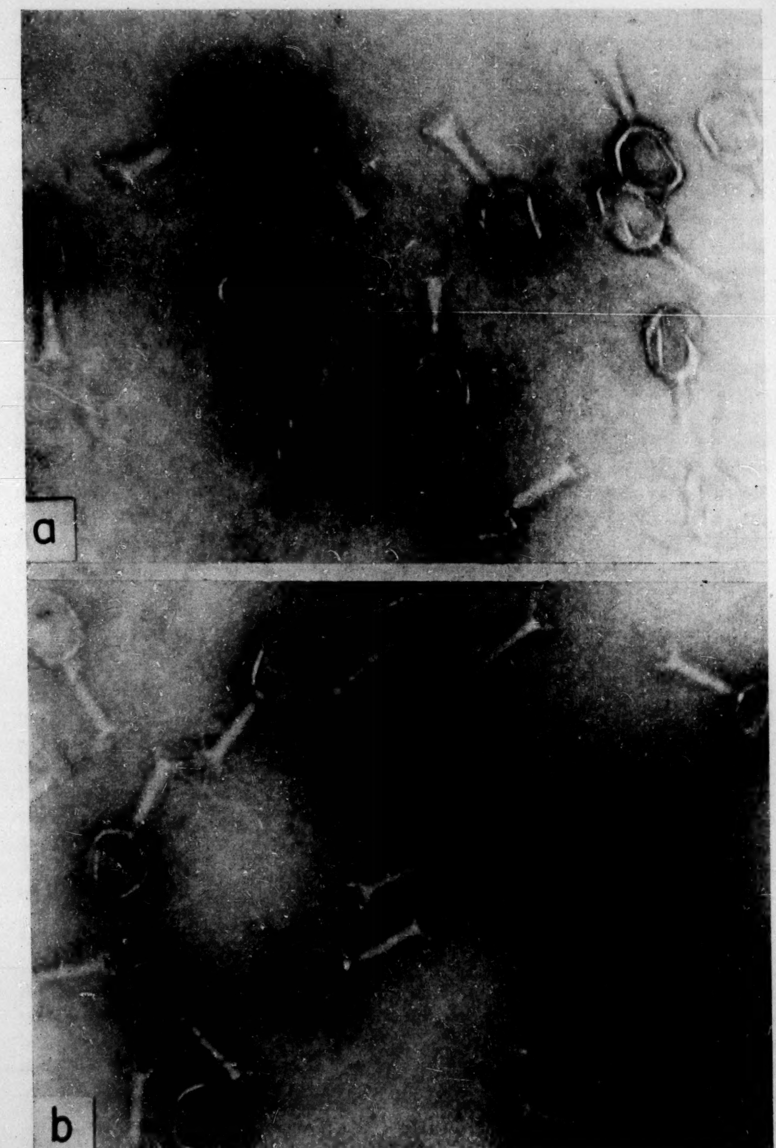

Fig. 2. Electron micrographs of the two forms of T2 "Ghosts" using the PTA procedure after formol fixation. The magnification is 100,000 × for both pictures. (a) T2 "ghosts" at pH 7.0; and (b) T2 ghosts at pH 5.7.

basis at 20°. The $D_{20,w}$ value of the form at pH 5.78 was found to be $3.40 \pm 0.10 \cdot 10^{-8}$ cm^2/sec and that of the form at pH 7.85 to be $2.35 \pm 0.09 \cdot 10^{-8}$ cm^2/sec. The ratio of $D5/D7$, 1.45 ± 0.07, calculated from these values is in good agreement with the ratio of 1.43 ± 0.05 obtained for $S5/S7$ (see later). Using the Svedberg formula and a partial specific volume of 0.66 ml/g (see ref. 12), the molecular weight of the fast form was found to be $214 \cdot 10^6$ and the slow form, $216 \cdot 10^6$. These data firmly place the cause of the dual sedimentation phenomenon of T2 on a change in individual particles

Morphological appearance of two phage forms

The respective sedimentation forms of whole T2 and T2 "ghosts" were fixed by the 2% formol treatment of BENDET, ALLISON AND LAUFFER[6] and examined in an RCA EMU-3 electron microscope. Both palladium shadowing and the PTA embedding method described by BRENNER AND HORNE[15] were used to increase contrast. Both methods showed that the slow form of whole T2 had a relatively longer head than the fast form. The axial ratio (length/width) of the head of over 200 individual particles in 4 separate experiments at pH 7.0 was 1.49 ± 0.07, while the axial ratio of the head of the form at pH 5.7 was 1.29 ± 0.05.

The dimensions of the head of the phage form after formol fixation in pH 7.0 buffer at 37° were 119 ± 4 mμ long by 80 ± 2 mμ wide. The face in the body of the head was 72 mμ long and apex height was 23 mμ. After fixation at pH 5.7 at 25°, the head was 103 ± 3 mμ long and was still 80 ± 2 mμ wide. The approximately 15 mμ shortening appeared to occur almost exclusively in the body of the head; the face of the short form was 58 mμ long as compared to 72 mμ in the longer form. No change was apparent in the height of the apices. Representative electron micrographs of these two forms of whole T2 are presented in Fig. 1.

Electron micrographs of the two forms which T2 ghosts (see later for sedimentation properties) assume are shown in Fig. 2. The head of the slower sedimenting form still largely retains the polyhedral shape of the phage particle although it deforms readily. The head has an axial ratio of 1.42 ± 0.05 and is 123 ± 5 mμ long by 85 ± mμ wide. The faster sedimenting form has a rounder head with an axial ratio of 1.22 ± 0.13 mμ although there is some variation in the size of the particles. The heads are 106 ± 10 mμ long by 86 ± 6 mμ wide. One important difference between the two forms is that the fast form has a band (indicating a higher concentration of protein in the phosphotungstic procedure) at least 13 mμ wide across the center of the head and normal to the tail.

The tails of all intact phage particles as well as phage ghosts have identical configurations. The fibers at the tail tips are extended in all forms. This can be readily seen in Fig. 2 of the ghosts. The tails are 125 mμ long when measured using either the phosphotungstic acid method or palladium shadowing after formol fixation. The tail width, however, was 20 mμ after metal shadowing but only 13 mμ in the phosphotungstic preparation.

These dimensions for the form of T2 at pH 7.0 are 25% larger than those reported by WILLIAMS AND FRASER[16] for metal shadowed frozen-dried T2, head 95 × 65 mμ and tail 100 mμ long. It can be calculated that the size of frozen-dried T2 corresponds to the expected water-free virus particles while the particle measured after formol fixation corresponds to the form which is known to contain 0.6 gram of H_2O/g of virus[5]. For example, the volume of T2 after freeze-drying is $2.55 \cdot 10^{-16}$ cm^3 and since dry T2 has a density of 1.5, this corresponds to a weight of $3.8 \cdot 10^{-16}$ g. This value is in good agreement with the particle dry-weight calculated from the diffusion and sedimentation coefficients of $3.6 \cdot 10^{-16}$ g *i.e.*, $215 \times 10^6/6 \times 10^{23}$. The volume of formol-fixed T2 is $4.5 \cdot 10^{-16}$ cm^3 and using the hydrated density of 1.26 the net weight of a T2 particle would be 5.7×10^{-16} *g*. This value, based on the dimensions in the electron micrographs, is in excellent agreement with that calculated from the physical measurements and the degree of hydration of $5.8 \cdot 10^{-16}$ g $[3.6 \times 10^{-16}\ (1 + .6) = 5.8\ 10^{-16}$ g].

Sedimentation analysis of whole T2

Analysis of the transition between the two sedimentation forms of T2 was performed in the Spinco Model E Ultracentrifuge. Virus concentrations of $2 \cdot 10^{12}$/ml were dialyzed for two days in sodium phosphate buffer, ionic strength 0.1, in the pH range of 5.78 to 7.54. At each pH studied, the temperature of the centrifuge rotor was varied over the range of 8 to 45°. It was found that the transition between the two forms of T2 was markedly dependent upon the temperature as well as the pH. At a particular pH, the fast form was transformed into the slow form as the temperature of the solution was increased. The $S_{20,w}$, temperature, and pH values are listed in Table I. The complete transition between the two forms occurred over a temperature range of about 13° at a given pH. Representative sedimentation diagrams are presented in Fig. 3, showing the effect of temperature at pH 7.00. The average sedimentation coefficient of the slow form was 710 ± 25 S and that of the fast form, 1017 ± 11 S. The ratio of S5/S7, 1.43 ± 0.05, is in agreement with the value of 1.42 reported by BENDET *et al.*[3].

The effect of temperature on the sedimentation rate of whole T2 in NaCl and $CaCl_2$ solvents of 0.1 ionic strength was also examined. Each of these solvents had a pH of about 6.3. As seen in Table II, temperature also had a decided effect on the

TABLE I

SEDIMENTATION RATES OF WHOLE T2 BACTERIOPHAGE IN PHOSPHATE BUFFER

pH 5.78		*pH 6.06*		*pH 6.25*		*pH 6.58*	
Temperature	$S_{20,w}$	*Temperature*	$S_{20,w}$	*Temperature*	$S_{20,w}$	*Temperature*	$S_{20,w}$
9.3	1024	8.0	1022	10.0	1028	8.0	1032
16.1	1020	14.7	1027	15.5	1022	16.2	1009
20.1	1007	22.0	1031	20.9	1012	21.7	1008, 757
29.9	1000	29.8	962	28.9	952	28.8	714
37.6	970	37.1	794	35.8	702	35.7	681
45.0	672	44.2	661	42.4	650		

pH 7.00		*pH 7.34*		*pH 7.54*	
Temperature	$S_{20,w}$	*Temperature*	$S_{20\ w}$	*Temperature*	$S_{20,w}$
8.3	1021	8.3	1027, 757	9.8	738
11.7	1000, 737	12.5	1014, 748	14.1	731
18.3	1000, 745	16.4	1021, 729	19.7	699
23.9	704	21.9	698	24.6	686
30.6	674	28.9	662	28.9	670

Temperatures in ° C.

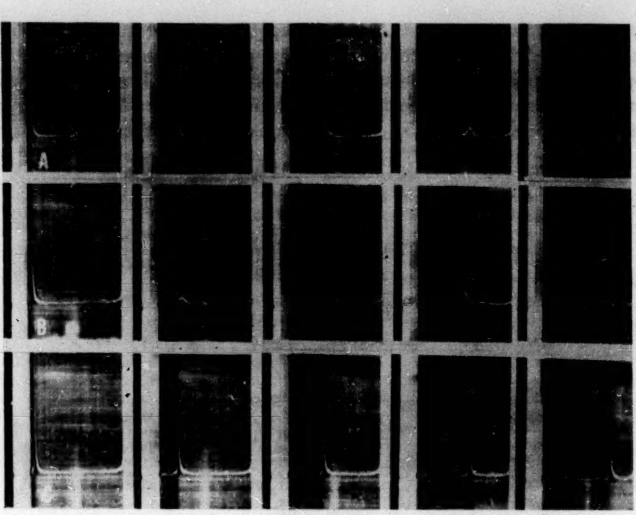

Fig. 3. Representative sedimentation diagrams of whole T2 showing the effect of temperature: (a) 15°, 747 S and 1016 S; (b) 22°, 742 S and 1063 S; and (c) 27°, 701 S.

TABLE II

SEDIMENTATION RATES OF WHOLE T2 BACTERIOPHAGE IN NaCl AND $CaCl_2$

In both cases the ionic strength was 0.1 and the pH was 6.3.

NaCl		*$CaCl_2$*	
Temperature °C	$S_{20,w}$	*Temperature* °C	$S_{20,w}$
19	1031	21	1094
25	1042, 773	27	1077
32	747	35	1086
40	704	42	1108

sedimentation rate of whole T2 in NaCl. This effect was similar to that obtained in sodium phosphate buffer. On the other hand, the presence of Ca^{++} appeared to keep the phage in the fast form even at temperatures where the phage in NaCl and sodium phosphate buffer was entirely in the slow form.

Sedimentation analysis of T2 "ghosts"

The sedimentation behavior of the protein coats of T2 was also studied as a function of pH and temperature. T2 "ghosts" at a concentration of $6 \cdot 10^{12}$/ml, were dialyzed for two days in sodium phosphate buffer, ionic strength, 0.1, in the pH range of 5.73 to 7.00. It was observed that T2 "ghosts" exhibit a dual sedimentation phenomenon similar to that of whole phage. The interconversion of the two forms of "ghosts" was markedly dependent upon the temperature as well as the pH of the solvent. The results are listed in Table III. From the data presented, it is evident that intermediate forms can exist. At values of pH and temperature where both forms are present, the sedimentation rate consistently approaches the limiting value obtained at the pH where only one form exists. This effect was not observed with whole T2. The limiting values of the sedimentation coefficients of the two forms of T2 "ghosts" were taken as 256 ± 1S and 166 ± 1S; the ratio of $S5/S7$ is then 1.54 ± 0.01.

TABLE III

SEDIMENTATION RATES OF T2 "GHOSTS" IN PHOSPHATE BUFFER

pH 5.73		*pH 5.79*		*pH 5.86*	
Temperature	$S_{20,w}$	*Temperature*	$S_{20,w}$	*Temperature*	$S_{20,w}$
12.6	257	14.6	250	15.1	248, 184
18.0	255	20.0	251, 188	20.0	248, 180
24.0	256	23.2	249, 181	24.1	177
		27.3	181	28.5	176
		32.3	180	33.4	176

pH 5.92		*pH 5.99*		*pH 6.21*	
Temperature	$S_{20,w}$	*Temperature*	$S_{20,w}$	*Temperature*	$S_{20,w}$
9.2	247, 181	9.8	241, 170	9.2	221, 164
13.2	251, 182	16.2	247, 175	15.8	226, 167
18.0	246, 178	21.4	171	21.7	167
22.0	175	27.4	172	27.2	167
29.0	174	33.0	171	32.8	166

Temperatures in ° C.

Kinetic analysis of the interconversion of the two viral forms

The thermodynamic changes occurring in individual virus particles were calculated from the relative concentrations of the two sedimentation forms at various temperatures. Using these data and invoking a general reaction of the type

$$S7 + nX \rightleftharpoons S5 \tag{1}$$

where n is the number of moles of X/mole of T2 required for the transition, one can evaluate the ΔF°, ΔH° and ΔS° values for the transition. Choosing the activating agent, X, of the transition was complicated by the fact that the transition was affected by both the buffer and the concentration of NaCl. Throughout this investigation and those of others previously mentioned, positively charged ions, particularly H^+ were observed to be directly concerned with the dual sedimentation phenomenon. For this reason, the role of H^+ was evaluated in detail and less attention was paid to Na^+ which also may have some effect.

As can be seen from equation (1), the equilibrium constant K is given by

$$K = \frac{(S5)}{(S7)\,(X)^n} \tag{2}$$

or,

$$-\log X = \frac{1}{n}\log\frac{(S7)}{(S5)} + \frac{1}{n}\log K \tag{3}$$

This is the equation of a straight line with a slope of $1/n$ and a y-intercept of $1/n \log K$. Eqn. (3) was utilized in obtaining values of n at different temperatures. Since the sedimentation peaks were quite sharp, the relative concentration of the two forms was easily determined from the peak heights and the temperature, T^*, at which equal concentrations occurred, could be estimated to about ± 1°. The data in Table IV for T2 "ghosts" illustrates the calculation; and values of n for whole T2 were obtained in a similar manner. (If the activating agent was assumed to be Na^+ instead of H^+ values of n about ten-fold larger were obtained.) Values for n at other temperatures were obtained by assuming a linear relationship between temperature and n. In order to incorporate as much of the data as possible, the temperatures chosen for calculating $\Delta F°$, $\Delta H°$, and $\Delta S°$ were those at which equal concentrations of $S7$ and $S5$ existed. These temperatures are given in Table V. $\Delta H°$ was determined, using absolute temperatures T^*, from the VAN 'T HOFF equation,

TABLE IV

EVALUATION OF THE NUMBER OF MOLES OF H^+ INVOLVED IN THE TRANSITION OF T2 GHOSTS

Temperature °C	*pH*	*S7/S5*	*n Moles of H+*
9.4	6.21	6/1	3.5
	5.99	6/7	
	5.22	2/3	
20	5.92	4/1	5.6
	5.86	8/5	
	5.79	8/11	

TABLE V

TEMPERATURE AT WHICH CONCENTRATIONS OF THE TWO FORMS OF T2 ARE EQUAL

The ionic strength was 0.1 in all experiments.

Whole T2			*T2 "Ghosts"*		
Solvent	*pH*	*Temperature °C*	*Solvent*	*pH*	*Temperature °C*
Phosphate	5.78	42	Phosphate	5.73	28
	6.06	37		5.79	22
	6.25	31		5.86	18
	6.58	24		5.92	15
	7.00	18		5.99	10
	7.34	9			
NaCl	6.3	25			
$CaCl_2$	6.3	>49			

$$\frac{d \ln K}{dT} = \frac{\Delta H^0}{RT^2}$$

If $\Delta H°$ is independent of temperature, a straight line relationship between $\log K$ and $1/T^*$ will result; this line will have a slope given by $-\Delta H°/2.303R$, from which $\Delta H°$ can be easily obtained. As seen in Fig. 4, a plot of $\log K$ *versus* $1/T^*$ yields the desired straight line for whole T2 and T2 "ghosts" using H^+ (or Na^+) as the activating agent in the transition. The other variables ΔF° and ΔS° were calculated utilizing the relations,

$$\Delta F^0 = - RT \ln K \tag{5}$$

and

$$\Delta S^0 = \frac{\Delta H^0 - \Delta F^0}{T} \tag{6}$$

A comparison of the thermodynamic values at 22° is given in Table VI. It can be seen from Tables V and VI that the slow to fast form transition is qualitatively the same for whole T2 and T2 "ghosts". However, the transition of the "ghosts" occurs over a much narrower pH range and involves much larger changes in the thermodynamic variables. It must be emphasized that this is a real difference and is not due to merely a difference in concentrations of whole phage and "ghosts" examined in the ultracentrifuge. The magnitude of ΔF°, ΔH° and ΔS° is not surprising because we are dealing with a reaction involving the entire molecular aggregate composing the head of T2 and not a simple molecular reaction.

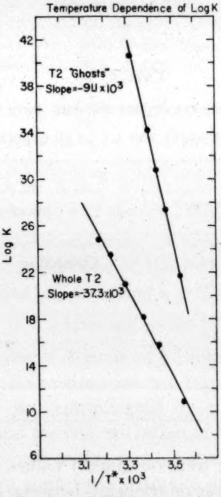

Fig. 4. The relationship between K, the equilibrium constant for the interconversion of the two forms of whole T2 or of T2 "ghosts", and $1/T^*$, the reciprocal of the absolute temperature at which the two forms exist in equal concentration.

TABLE VI

THERMODYNAMIC VALUES FOR THE SLOW TO FAST TRANSITION AT 22°

	Activating agent	*ΔF° kcal/mole*	*ΔH° kcal/mole*	*ΔS° e.u.*	*$n(H^+)$*
T2 "ghosts"	H^+	—47.0	417	1573	6.0
Whole T2	H^+	—23.4	171	659	2.6

DISCUSSION

It is clear that the major morphological change involved in the dual sedimentation phenomenon of bacteriophage T2 is a variation in the dimensions of the head structure. Electron micrographs obtained by two different procedures show that the slow form has a 15 % longer head than the fast form; this change in length occurs mainly in the body and immeasurably in the apices of the head. No other difference in appearance of the two forms was noted. That this variation in length of the head is due to a configurational change occurring in the head protein is demonstrated by the fact that protein coats or "ghosts" of T2 also exhibit two sedimentation coefficients. The main difference between whole phage and "ghosts" is that the latter has no DNA inside the protein coat. If the DNA were completely ineffective in the transition between the two forms, the estimates of $\Delta F°$, $\Delta H°$ and $\Delta S°$, and n, the number of activating protons, obtained would be the same with or without the DNA. The fact that they are not suggests that while the protein coat alone can undergo a change in length, DNA has some auxiliary action. This is also indicated by the intermediate values of $S_{20,w}$ obtained for T2 "ghosts". Apparently, when DNA is inside the phage head, the transition from one form to the other goes as an all-or-none reaction; when the DNA is removed, the dimensions of the head can change gradually until an unstable state is reached and the head then shifts rapidly to its limiting length.

The bonds being broken in the configurational change in the head structure must be weak, since the two forms are readily interchanged[3]. This implies that there must be a small change in heat content and entropy per bond broken and formed. The high values of $\Delta H°$ and $\Delta S°$ calculated for T2 "ghosts" indicate that a great many of these non-covalent bonds are being broken. While a large number of weak bonds are involved in the transition, the data indicated that relatively few positively charged ions are required to initiate the reactions and that probably the reaction is completed by some cooperative action of the protein subunits composing the viral head.

The main arguments for rejecting the contention of BENDET *et al.*[3,6] that the anomalous sedimentation and diffusion behavior of T2 was due to a difference in the configuration of the tail fibers are as follows: (a) Using procedures indentical to those of BENDET *et al.*[6], no difference in tail fiber configuration was observed, but changes in head shape were apparent. Moreover, the extended tail fibers shown by BENDET and his colleagues have not been observed after formol fixation by HERRIOT AND BARLOW[13] or by KELLENBERGER AND ARBER[17]. (b) The sedimentation properties of T2 "ghosts" are not in accord with BENDET'S hypothesis. Removal of the DNA eliminates the density heterogeneity of these particles and greatly weakens any assumption of preferred orientation. Further, if the tail fiber model were correct, the number of activating ions required for the conversion of the slow to fast forms should be the same for whole T2 and "ghosts"; in contrast to this expectation, "ghosts" required 2 to 3 times as many activating ions as whole phage. (c) The tail fiber model was conceived to account for the apparent discrepancy in the ratios of $D5/D7$ and $S5/S7$. As shown in this paper, this discrepancy does not exist. LAUFFER'S group[3,5] did not appreciate the effect of temperature on the transition between the two forms. At the pH and temperature used in their experiments, the phage could not have been maintained in the slow form. Hence, the ratio of $D5/D7$, 1.24, calculated from their results is in error. This effect of temperature also explains the different values for the transition limits of pH reported in the literature. SHARP *et al.*[2] observed that above pH 5.8, only the slow form existed. However, LESLEY, FRENCH AND GRAHAM [18] obtained the fast form at pH 6.05. SINGER AND SIEGAL[19] found that the fast form existed at pH values as high as 6.9. In the course of their studies, BENDET *et al.*[3] indicated that the interconversion of the fast and slow forms of whole T2 covered about one-half a pH unit, with a central value at about pH 6.1. These apparent discrepancies in the effect of pH disappear when the role of temperature is considered.

When an attempt is made to account quantitatively for the differences in the diffusion and sedimentation coefficients of the two forms of T2 due to the change in head length, the importance of the change in the effective volume, V_e, is soon realized. The usual practice in relating the diffusion coefficient of a particle to its hydrodynamic shape is to use the EINSTEIN-SUTHERLAND equation, $D = kT/f$, where f is the frictional coefficient. According to ONCLEY[20], the frictional coefficient is a function of both the geometric dimensions and the effective volume, V_e. SCHERAGA AND MANDELKERN[21] modified the view that the effective volume is solely a function of the anhydrous partial specific volume and hydration by pointing out that this view neglects possible flow of solvent through the particle domain, deviation of the shape of the domain from that of an ellipsoid of revolution and deformation of the domain. With these modifications in mind, the frictional coefficient is,

$$f = 6\pi\eta\left(\frac{3V_e}{4\pi}\right)^{1/3}\left(\frac{f}{f_0}\right)_{\text{geom.}} \tag{7}$$

The shape dependent frictional coefficient can be calculated by assuming that the particle in question may be approximated by an equivalent ellipsoid of revolution. Using the equation derived by GANS[22] for randomly oriented prolate ellipsoids,

$$f_{\text{geom.}} = \frac{12\pi\eta\sqrt{b^2 - a^2}}{\ln\dfrac{b + \sqrt{b^2 - a^2}}{b - \sqrt{b^2 - a^2}}} \tag{8}$$

the shape dependent frictional coefficients of the fast and slow forms of T2 were calculated. The ratio $(f7/f5)_{\text{geom.}}$ was found to be 1.08. Assuming that the effective volumes of the two forms are unknown, their ratio may be determined from the ratio of $S5/S7$, 1.43 ± 0.05, obtained experimentally.

$$\frac{S5}{S7} = \frac{f7}{f5} = \left(\frac{V_{e}7}{V_{e}5}\right)^{1/3}\left(\frac{f7}{f5}\right)_{\text{geom.}} \tag{9}$$

The ratio of the effective volumes of the slow and fast forms, $Ve7/Ve5$, calculated in this manner was 2.3 ± 0.3. On the other hand, the ratio of the volumes determined from the dimensions of the two forms was found to be 1.24. This discrepancy may be partly due to the fact that the shape dependent frictional coefficient for ellipsoids is relatively insensitive to changes in the axial ratio. This inherent property tends to minimize the value of $(f7/f5)_{\text{geom.}}$ which in turn exaggerates the value of $Ve7/Ve5$ obtained from eqn. (9). Whether a tadpole-shaped object such as T2 phage can be approximated by an equivalent ellipsoid of revolution is itself debatable.

As suggested above, the problem of solvent flow through the particle, which could affect the hydroxynamic behavior of T2, must be considered. Two lines of evidence point towards the conclusion that the two head forms of T2 behave differently with respect to solvent flow. ANDERSON, RAPPAPORT AND MUSCATINE[23] found that the head of the closely related bacteriophage T6 could exist in two osmotic shock forms. At a temperature of 42° and higher, this phage was found to be in a form resistant to osmotic shock. This finding correlates well with our results on the effect of temperature on the sedimentation properties of T2; it is highly probable that the osmotic shock resistant form is the slower sedimenting, long head form. Since susceptibility to osmotic shock is a function of the relative differences in permeability between solute and water, the long head form is more permeable to the flow of solvent than the short head, osmotic shock sensitive, form. In the studies on the sedimentation properties of T2 "ghost", it was found that the ratio of $S5/S7$ was 1.54, whereas the same ratio for whole phage was 1.43. This again suggests that the long head form is more permeable to solvent since removing the DNA would tend to enhance this difference. The direction of the change in $S5/S7$ indicates that the difference in solvent flow would decrease the relative sedimentation rate of the long head form.

With regard to deformation, SCHACHMAN[24] has stated that the intramolecular organization of a particle could vary, without a decrease in molecular weight, causing a drastic change in the effective volume but little change in the dimensional volume. That such a mechanism is operating in T2 is evidenced by the fact that the head dimensions of this particle do indeed change, without any change in the molecular weight. Thus it is seen that the apparent discrepancy between the effective volume and dimensional volume ratios can be accounted for by the anomalous behavior of the voluminous head of this virus. Furthermore, all these considerations support the conclusion that the sedimentation and diffusion properties of bacteriophage T2 are directly related to configurational changes occurring in the head protein.

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REFERENCES

[1] A. E. HOOK, D. BEARD, A. R. TAYLOR, D. G. SHARP AND J. W. BEARD, *J. Biol. Chem.*, 165 (1946) 241.
[2] D. G. SHARP, A. E. HOOK, A. R. TAYLOR, D. BEARD AND J. W. BEARD, *J. Biol. Chem.*, 165 (1946) 259.
[3] I. J. BENDET, L. G. SWABY AND M. A. LAUFFER, *Biochim. Biophys. Acta*, 25 (1957) 252.
[4] F. W. PUTNAM, *J. Biol. Chem.*, 190 (1951) 61.
[5] N. W. TAYLOR, H. T. EPSTEIN AND M. A. LAUFFER, *J. Am. Chem. Soc.*, 77 (1955) 1270.
[6] I. J. BENDET, J. L. ALLISON AND M. A. LAUFFER, *Virology*, 6 (1958) 571.
[7] M. H. ADAMS, *Methods in Medical Research II*, Yearbook Publishers, Inc., Chicago, 1948, p. 1.
[8] R. M. HERRIOTT AND J. L. BARLOW, *J. Gen. Physiol.*, 40 (1957) 809.
[9] S. S. COHEN AND T. F. ANDERSON, *J. Exptl. Med.*, 84 (1946) 511.
[10] M. SCHLESINGER, *Biochem. Z.*, 273 (1934) 306.
[11] L. M. KOZLOFF AND F. W. PUTNAM, *J. Biol. Chem.*, 181 (1949) 207.
[12] A. R. TAYLOR, *J. Biol. Chem.*, 165 (1946) 271.
[13] R. M. HERRIOTT AND J. L. BARLOW, *J. Gen. Physiol.*, 36 (1952) 17.
[14] H. K. SCHACHMAN, *Methods in Enzymology IV*, Academic Press, New York 1957, p. 32.
[15] S. BRENNER AND R. W. HORNE, *Biochim. Biophys. Acta*, 34 (1959) 103.
[16] R. C. WILLIAMS AND D. FRASER, *J. Bacteriol.*, 66 (1953) 458.

[17] E. Kellenberger and W. Arber, *Z. Naturforsch.*, 10b (1955) 698.
[18] S. M. Lesley, R. C. French and A. F. Graham, *Can. J. Research, E* 28 (1950) 281.
[19] S. J. Singer and A. Siegal, *Science*, 112 (1950) 107.
[20] J. L. Oncley, *Ann. N.Y. Acad. Sci.*, 41 (1941) 121.
[21] H. A. Scheraga and L. Mandelkern, *J. Am. Chem. Soc.*, 75 (1953) 179.
[22] R. Gans, *Ann. Physik*, 86 (1928) 628.
[23] T. F. Anderson, C. Rappaport and N. A. Muscatine, *Ann. Institute Pasteur*, 84 (1953) 5.
[24] H. K. Schachman, *Ultracentrifugation in Biochemistry*, Academic Press, New York, 1959, p. 215.

PATTERNS OF CELLULAR CONTROLS OPERATING IN BACTERIOPHAGE REPRODUCTION

I. EFFECT OF 5-FLUOROURACIL ON THE MULTIPLICATION OF SEVERAL COLIPHAGES

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SUMMARY

The effects of 5-fluorouracil on bacteriophage production in *E. coli*, strain B, have been studied. In the presence of the fluoropyrimidine the phage yields are less than 0.5 % of the control in T2r infection, whereas as much as 8 % of the control yield is obtained in T3 infection. The addition of uracil does not affect the inhibition of T2r multiplication, but restores T3 formation to 40–50 % of normal. Supplementation with thymidine has little effect on T3, but raises the yield of T2r to more than one fifth of the control. Essentially complete restoration of phage reproduction is observed when both uracil and thymidine are present together with fluorouracil.

Orienting experiments with phages T1 and T4r are also described.

INTRODUCTION

The nature of the control mechanisms that guarantee the unaltered reproduction of the various high polymers of the cell is among the most important problems in chemical biology[1]. There are many ways in which the study of this question may be undertaken, just as there must exist many such cellular controls; but it is clear that no direct approach to their description is discernible at present. We have, in the recent past, repeatedly discussed the conceptual basis of the problem[1–3] and attempted to define it through experimental studies[4–6]. Of particular interest, in this connection,

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are those chemical agents that, by interfering with the strict coordination of the synthetic abilities of the cell, are able to disconnect the reproduction of one cellular polymer from that of the others. One such "uncoupling" agent, 5-fluorouracil[7], is studied in this and the following communication[8] in its effect on bacteriophage multiplication.

The action of this fluoropyrimidine on the synthesis of protein and nucleic acid in various strains of *E. coli* has been studied previously in this laboratory. In a uracil-requiring mutant, the substitution of fluorouracil for uracil has been shown to inhibit the formation of deoxyribonucleic acid and almost entirely that of ribonucleic acid, but to permit a considerable increase in protein[5]. The synthesis of several constitutive enzymes continued in the presence of the agent, but no formation of inducible enzymes could be observed[5,9]. In *E. coli*, strain B, as well as in the uracil auxotroph in the presence of uracil, 5-fluorouracil inhibited the synthesis of deoxyribonucleic but not of ribonucleic acid; it was even found to be incorporated into the latter in a considerable quantity[10].

The shunt in the cellular controls attendant upon the infection of a cell by a bacteriophage particle or a virus is far-reaching in its consequences. New protein and nucleic acid molecules are produced and there must become operative several enzymes that were either dormant or non-existent in the host. It may be expected that much could be learned about the nature of the normal cellular control mechanisms through a study of the effect of uncoupling agents on the events leading to phage multiplication. In view of the observations on 5-fluorouracil mentioned before, it appeared of interest to compare the effect of this agent on the formation of the even-numbered coliphages of the T series, which require the synthesis of a new nucleic acid component, 5-hydroxymethylcytosine[11], and of the odd-numbered phages, in which no such requirement is apparent.

MATERIALS AND METHODS

5-Fluorouracil was obtained through the courtesy of Dr. J. A. AESCHLIMANN, Hoffmann-La Roche Inc., Nutley, New Jersey. Uracil, 5-bromouracil, thymine and thymidine were commercial preparations of checked purity.

The bacterial strain used in all studies was *E. coli* B which is sensitive to the T phages. The bacteriophage stocks used were T1, T2r, T3 and T4r; they were kindly given us by Prof. F. J. RYAN of this University. The methods used for the preparation of phage stocks of high titer and for the assay of infective particles were adapted from the literature[12].

For each experiment, cells from an overnight culture in synthetic medium[13] or Difco "Penassay" broth were inoculated into tubes containing 5 ml of the particular medium. The tubes were incubated at 37°, with aeration, until an O.D. (read in a Klett-Summerson colorimeter with the use of the 660-mμ-filter) corresponding to $2 \cdot 10^8$ cells per ml was reached. At this time, the various supplements were added to the cultures, as noted in the description of the experiments, and the tubes reincubated. 30 min later, when no further increase in the turbidity of the cultures containing only fluorouracil was noticeable, phage were added. Turbidity readings were taken at intervals until no further changes were noted. The lysates were assayed for plaque-forming units after being kept at 4° overnight.

RESULTS AND DISCUSSION

Inhibition of lysis

The effects of fluorouracil on phage growth in the synthetic medium are shown in Fig. 1. It is apparent that phage production, as measured by the loss of turbidity of infected cultures, is inhibited only in the even-numbered strain tested. It was found that the multiplicity of infection used, affected the shape of the lysis curves. In cultures infected with T3, a decreasing multiplicity of infection resulted only

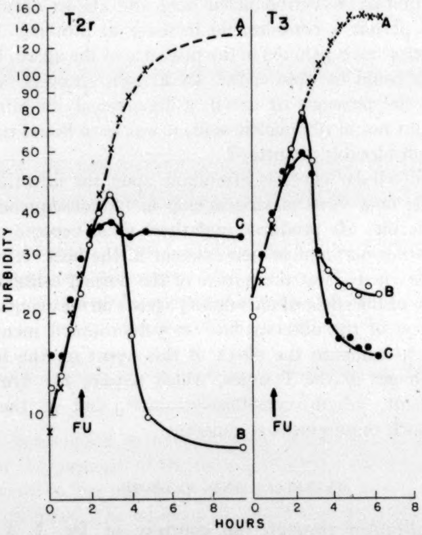

Fig. 1. Effect of 5-fluorouracil on bacterial lysis by phage. FU (50 μg/ml) was added to logarithmically growing cultures of *E. coli* B at the time indicated by arrows. 30 min later, each culture was infected at a ratio of approx. 1 phage particle per 10 bacteria. A, ×—×, control; B, ○—○, infected in absence of 5-fluorouracil; C, ●—●, infected in presence of 5-fluorouracil.

TABLE I

EFFECT OF 5-FLUOROURACIL ON MULTIPLICATION OF T2r

In each experiment 50 μg of 5-fluorouracil/ml of logarithmically growing culture of *E. coli* were employed. 0.5 h later, phage were added to the cultures. At completion of lysis, cells and debris were removed by centrifugation and the supernatant fluids were assayed for plaque-forming units.

Phage particles per ml (as multiples of 10^7)			*Yield ratio 5-fluorouracil/control*
	Yield		
Input	*Control (without 5-fluorouracil)*	*With 5-fluorouracil*	
6	220	1	0.004
60	140	0.7	0.005

in an increase in the time needed for the onset of lysis. On the other hand, infection with a high multiplicity of T2r produced lysis, whereas a low multiplicity of infection resulted in the complete inhibition of lysis. In order to test the production of infective particles under conditions of multiple infection with T2r, samples of a logarithmically growing culture were infected at two different ratios of phage to bacteria, namely, 1:5 and 2:1. Table I indicates that the lysis caused by multiple infection with T2r in the presence of fluorouracil is not accompanied by the release of infective particles.

Since under these conditions the lysis of a cell does not release infective phage, a noticeable drop in turbidity is not to be expected when only a small proportion of the cell population is infected with T2r. If, on the other hand, the first cells to be infected do give rise to infective particles, these will infect the remainder of the population and cause visible lysis regardless of whether new phage particles are or are not synthesized in this second cycle of infection. In all subsequent experiments on lysis, infection was performed at a ratio of one phage per ten bacteria so that less than 10 % of the cells would be infected in the first cycle.

In order to test the effects of the duration of fluorouracil treatment, the substance was added (a) 10 min before the addition of phage, (b) together with the phage, or (c) 12 min after the addition of phage. Fig 2 indicates that the effect of fluorouracil on lysis induced by T3 is only due to the depression of cell growth. In cultures infected with T2r—although fluorouracil completely inhibits lysis when added simultaneously with phage or before phage addition—some lysis does occur when the compound is added after the phage. This interval—12 min after infection—is about the time of appearance of the first intracellular phage particles[14,15]; and this may be taken to

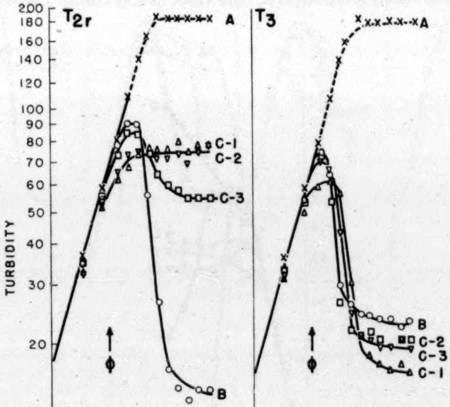

Fig. 2. Effects of duration of treatment with 5-fluorouracil. A, ×—×, control; B, ○—○, infected in absence of 5-fluorouracil; C, 50 µg/ml 5-fluorouracil added: △—△, 10 min before infection; ▽—▽, at time of infection; □—□, 12 min after infection.

mean that fluorouracil inhibits the formation of phage rather than its maturation or release from the cell. The difference between the effects on T3 and T2r is not due simply to the shorter latent period of the former, since the addition of fluorouracil to cells as much as 60 min before infection with T3 does not result in the inhibition of lysis.

Reversal of lysis inhibition in T2r infection

One of the effects of 5-fluorouracil appears to be the interference with the enzymic synthesis of thymidylic acid[7,16]. If the block to phage formation were due solely to the inhibition of the production of this nucleotide, supplementation of the medium with thymine or thymidine might be expected to abolish the inhibition. This was not found to be the case. Cells infected with T2r in synthetic medium, in the presence of fluorouracil, required the presence of both thymidine and uracil in order to exhibit lysis (Fig. 3). Several concentrations of uracil up to 20 μg/ml and of thymidine up to 50 μg/ml were tested. The combination of 20 μg of uracil and 40 μg of thymidine/ml was the lowest concentration to support full lysis. Thymidine alone, however, in a concentration as high as 100 μg/ml, was ineffective in overcoming lysis inhibition. It is of interest to note that thymidine alone has no effect on the turbidity of a culture treated with fluorouracil, whereas uracil alone causes an appreciable

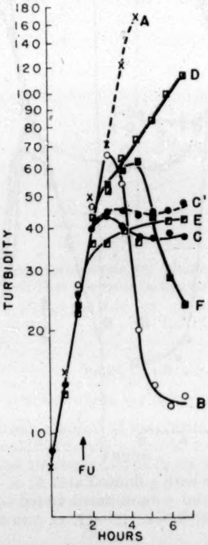

Fig. 3. Reversal of lysis inhibition in T2r infection. A, ×—×, control; B, ○—○, cells infected in absence of pyrimidines; C, ●—●, cells infected in presence of 5-fluorouracil; C′, ●—●, uninfected cells in presence of 5-fluorouracil; D, ◪—◪, cells infected in presence of 5-fluorouracil and uracil; E, ◩—◩, infection in presence of 5-fluorouracil and thymidine; F, ■—■, infection in presence of 5-fluorouracil, uracil and thymidine. Concentrations of pyrimidines per ml: 50 μg 5-fluorouracil; 20 μg uracil; 50 μg thymidine. Time of addition of 5-fluorouracil indicated by arrow; phage added 30 min later.

increase in turbidity (Fig. 3). Thymine can replace thymidine, but slightly higher concentrations of the free pyrimidine are necessary. 5-Bromouracil also showed limited activity as a reversing agent: it could replace thymidine in abolishing the inhibiting action of fluorouracil on lysis and was partially effective in supporting phage growth in the presence of fluorouracil.

The effects of uracil and thymidine on cells treated with fluorouracil appear to be different: both are needed for the full production of phage. We proceeded, therefore to stagger the order and the periods of supplementation. In all cases, actively growing cells received fluorouracil (50 μg/ml) 0.5 h before the addition of phage. The cells then were divided into three series of cultures. To the first series, uracil and thymidine were added simultaneously with fluorouracil or 15, 30, 60 min after the inhibitor. The second series received fluorouracil and thymidine at the same time, and only uracil was added at the noted intervals. In the third series, fluorouracil and uracil were added together, the supplementation with thymidine being staggered. It will be gathered from Fig. 4 that the effects differ. It is clear that in the order of events uracil comes before thymidine. In the presence of uracil, no appreciable delay in lysis is caused by later supplementation with thymidine. In the presence of thymidine, the delayed addition of uracil approximates the effects of the simultaneous addition of both supplements at the various times indicated.

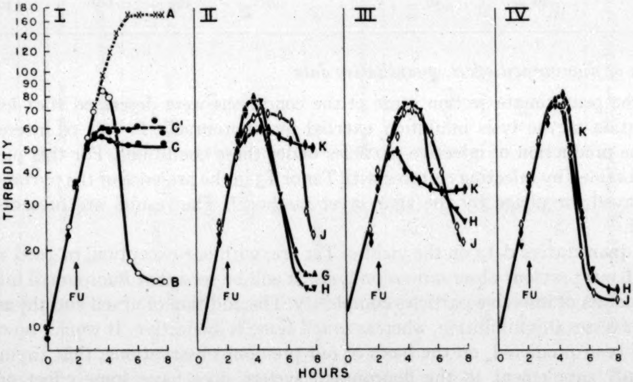

Fig. 4. Effects of delayed addition of supplements on lysis induced by T2r. 50 μg/ml 5-fluorouracil added at time indicated by arrows. Phage added at a ratio of 1 phage per 10 bacteria 30 min after 5-fluorouracil addition. Turbidity curves: I, no supplements present. II, delayed addition of uracil (20 μg/ml) plus thymidine (50 μg/ml). III, delayed addition of uracil to cells treated simultaneously with 5-fluoroucil and thymidine IV, delayed addition of thymidine to cells treated simultaneously with 5-fluorouracil and uracil. A, ×—×, uninfected control, B, ○—○, infection in absence of pyrimidines; C, ●—●, infection in presence of 5-fluorouracil; C′, ●—●, uninfected cells in presence of 5-fluorouracil; G, ▲—▲, supplements added simultaneously with 5-fluorouracil; H, ▼—▼, supplements added 15 min after 5-fluorouracil; J, ◇—◇, supplements added 30 min after 5-fluorouracil. K, ◆—◆, supplements added 60 min after 5-fluorouracil.

Viability of cells

In order to test the effect of the various supplements in the concentrations used here on the viability of noninfected cells, fluorouracil was added to logarithmically growing cultures in the synthetic medium simultaneously with either thymidine or uracil or both. Samples of the cultures were removed at intervals, diluted with distilled water and plated on "Penassay" plates. The results are presented in Table II. Fluorouracil has a bactericidal effect which is partially abolished by uracil; thymidine allows some increase in viable cells, whereas both pyrimidines are required for normal increase.

TABLE II

EFFECTS OF SUPPLEMENTS ON VIABILITY OF *E. coli* CELLS

Supplements (μg/ml)			*Viable cells (per ml) × 10^{-6}, after treatment (in minutes)*				
Fluorouracil	*Uracil*	*Thymidine*	*0.*	*30*	*60*	*120*	*320*
0	0	0	200	330	530	1400	5400
50	0	0		160	140	26	1.5
50	20	0		270	220	84	54
50	0	50		280	260	230	530
50	20	50		200	360	690	3300

Abolition of fluorouracil effect: quantitative data

In the penultimate section some of the conditions were described that lead to the abolition of the lysis inhibition exerted by fluorouracil. It was of interest to follow the production of infective particles under these conditions. For this purpose lysates obtained by infecting cultures with T2r or T3 in the presence of the pyrimidines were assayed for phage by the agar layer method[12]. The results are presented in Table III.

The quantitative data on the yield of T2r are, with one exception, in good agreement with our previous observations on lysis. It will be seen that fluorouracil inhibits the production of infective particles completely. The addition of uracil and thymidine entirely reverses this inhibition, whereas uracil alone is ineffective. It would, however, not have been predicted, on the basis of our previous observations, that thymidine as the only supplement to the fluorouracil system does have some effect on the inhibition. Under conditions of low phage input, similar to those followed in the experiments in Fig. 3, it restores phage production only to about 3% of the control value, but with multiple infection the phage yield is about $^1/_5$ of that of the control. The presence of fluorouracil was not found to inhibit the adsorption of phage appreciably, only 1 to 2% of the phage input remaining unadsorbed after 5 min under all conditions tested. It was also found that the various pyrimidines, alone and in combination, are without effect on the free phage.

Phage T3 is inhibited, but to a much lesser extent than is T2r by fluorouracil. Addition of uracil results in the production of about one-half the normal phage yield, whereas supplementation with both thymidine and uracil abolishes the inhibition almost completely. Thymidine alone appears to have no effect when all cells are infected in the first cycle of infection, but increases the phage yield when only a small proportion of cells are infected originally.

It has been suggested that the inhibitory effect of fluorouracil on *Lactobacillus leichmanii* is reversed non-competitively by thymine or thymidine and competitively by uracil[7]. We tested the effects of varying concentrations of these compounds on

TABLE III.

EFFECT OF 5-FLUOROURACIL AND OF REVERSING AGENTS ON PRODUCTION OF PHAGES T1, T2r, T3, AND T4r*

Supplements (µg/ml)			*T1*		*T2r*		*T3*		*T4r***	
Fluorouracil	*Uracil*	*Thymidine*	*Low input*	*High input*	*Low input*	*High input*	*Low input*	*High input*	*Low input*	*High input*
			*Relative phage yield****							
0	0	0	100 ($2.9 \cdot 10^{10}$)	100 ($1.4 \cdot 10^{10}$)	100 ($2.1 \cdot 10^{10}$)	100 ($1.6 \cdot 10^{10}$)	100 ($1.2 \cdot 10^{10}$)	100 ($1.8 \cdot 10^{10}$)	100 ($3.1 \cdot 10^{11}$)	100 ($8.7 \cdot 10^{10}$)
50	0	0	0.002	0.71	0.011	0.49	1.3	8.3	0.0003	0.054
50	20	0	0.45	1.63	0.013	0.61	49	43	0.0005	0.123
50	0	50	13	6.3	2.8	22	7.3	7.8	0.062	0.78
50	20	50	100	64	156	141	91	96	17	70
			Phage particles per viable cell §							
0	0	0	54	44	40	49	23	55	585	167
50	0	0	0.0041	0.64	0.016	0.49	1.1	9.4	0.0067	0.16
50	20	0	0.59	0.89	0.012	0.36	27	29	0.0071	0.22
50	0	50	14	3.2	2.3	13	3.3	5	0.74	1.3
50	20	50	80	46	91	113	30	87	146	165

* Cell density approximately $2 \cdot 10^8$/ml at the time of addition of supplements. Phage added 30 min later; "low input" less than 1 phage/2 cells; "high input" more than 2 phage/cell.

** 100 µg/ml tryptophan added to permit adsorption.

*** Relative yields represent means of three independent experiments, with the control yields (actual phage titers in parentheses) taken as 100.

§ Titer of phage divided by the number of viable cells at time of infection (see Table II). It was assumed that infection occurs 30 min after supplement addition at high phage input, 60 min at low input.

phage T2r production. The results of one experimental series are shown in Table IV. It will be seen that in the presence of 50 μg/ml of fluorouracil, thymidine as the only supplement, regardless of its quantity, permitted only 2% of the phage yield of the control. Thymine alone was much less effective than thymidine, but only slightly less so when compared with the nucleoside in the presence of uracil. In systems supplemented with uracil the phage yield increased very noticeably with increasing concentrations of thymidine or thymine. A limited, but definite, effectiveness of 5-bromouracil in overcoming the inhibitory action of the fluoro compound is also evident.

TABLE IV

T2r PRODUCTION IN PRESENCE OF 5-FLUOROURACIL AS A FUNCTION OF DIFFERENT SUPPLEMENT CONCENTRATIONS*

Supplements (μg/ml)					*Phage per ml* ($\times 10^{-7}$)
5-Fuorouracil	*Uracil*	*Thymidine*	*Thymine*	*5-Bromouracil*	
0	0	0	0	0	580
50	0	0	0	0	0.013
50	0	20	0	0	9.0
50	0	50	0	0	12
50	0	100	0	0	11
50	0	0	20	0	0.60
50	0	0	50	0	4.4
50	0	0	0	50	0.64
50	20	20	0	0	79
50	20	50	0	0	930
50	20	0	20	0	66
50	20	0	50	0	530
50	20	0	0	50	61

* All supplements were added 30 min before infection with 1 phage per 10 bacteria.

In an additional series of experiments the competitive reversal by uracil alone at different levels of fluorouracil was examined. At concentrations of 10 or 20 μg/ml of the inhibitor, equal quantities of uracil permitted a partial restoration of phage production (3% or more of the control yield). At the fluorouracil level employed in most of the present studies, 50 μg/ml, almost no restitution by uracil as the only supplement was observed.

Effect of 5-fluorouracil on other phages

In view of the remarkable difference in the effectiveness of fluorouracil in inhibiting the production of T2r and T3 phages, it was of interest to obtain orienting quantitative data on some other members of the T series. The effects of fluorouracil and of the various supplements on the multiplication of phages T1 and T4r are also included in Table III. Although large quantitative differences in the response of the various phage strains to the supplements are apparent, the findings are consistent qualitatively. Fluorouracil inhibits the production of all the phage strains tested to some extent: most markedly that of the even phages, least of T3. Phage T1 appears to be intermediate. Supplementation with thymidine alone partially reverses the inhibition; the addition of both thymidine and uracil greatly stimulates the production of all phages. The effect of uracil alone differentiates between the odd and the even phages tested. In the case of T2r and T4r there is no stimulatory effect by uracil, whereas the production of the odd-numbered phages, especially T3, is markedly stimulated by this pyrimidine.

Additional remarks

The order of events taking place during the "eclipse" period of infectivity, when the reorganization of the synthetic apparatus of the host brings about the production of viral components is less well known than some of the events themselves[17]. Among these there appears to be, at any rate in the case of T2r, the synthesis of new proteins and of a small amount of a specific ribonucleic acid. It was, in the light of our previous findings on the effects of 5-fluorouracil on *E. coli*[5,9,10], not without interest to compare the effects of this pyrimidine on the multiplication of some of the even- and odd-numbered T phages.

5-Fluorouracil appears to exert at least two distinguishable effects on the cell: it interferes with the normal synthesis of ribonucleic acid[18] and inhibits the formation of thymidylic acid[16,18]. To take the specific case of *E. coli* B, the host of the phages under consideration here: in the presence of the fluoro compound, the synthesis of deoxyribonucleic acid is blocked completely while that of protein and ribonucleic acid continues[9]. The addition of uracil to the inhibition system results in a stimulation of the formation of the two polymers mentioned last, but not of deoxyribonucleic acid; that of thymine, on the other hand, permits the resumption of deoxyribonucleic acid synthesis to a very limited extent without affecting the synthetic rates of the other compounds. Full supplementation (uracil and thymine) largely overcomes all blocks.

The findings described in the following paper[8] do, in fact, indicate that 5-fluorouracil blocks the synthesis of deoxyribonucleic acid completely in cells infected with $T2r^{+}$. Uracil is without effect on this inhibition; thymidine permits synthesis, but at a considerably slower than normal rate. When the system containing the fluoro compound is supplemented with both uracil and thymidine the degree of formation of deoxyribonucleic acid equals, or even surpasses, that of the controls.

In view of the great differences between phages T2r and T3 as regards the respective origins and quantities of required precursor material[17], a difference in the effects of 5-fluorouracil was to be expected; and this is borne out by the findings presented here. Different phages are affected differently. The multiplication of phage T2r is paralyzed most efficiently by the fluoro compound; an inhibition that is partially abolished by the simultaneous presence of thymidine, but not at all by uracil alone (Table III). According to the few results available for T4r the trend seems similar, but this phage is even more sensitive to the inhibitor. In contrast, phage T3 is much less affected by fluorouracil and rehabilitated to a large extent by uracil alone, but not by thymidine. In its response to inhibitor and reversal agents phage T1 is more similar to the even-numbered phages than to T3. Full supplementation with both uracil and thymidine largely abolishes the inhibition of all phages.

If one were to distinguish between a "uracil step" and a "thymidine step" in the pattern of the reversal of the fluorouracil effects by pyrimidine supplements, the results summarized in Table III and in Fig. 4, taken together with the findings presented in the following paper[8], would seem to indicate that the first step is concerned with the synthesis of "initial protein" (including newly arising enzymes), the second with that of deoxyribonucleic acid. In phages requiring the massive formation of the latter compound (*e.g.*, T2r and T4) uracil is ineffective, presumably because it is unable to reinstate the capacity of synthesizing thymidylic acid. On the other hand, thymidine as the only supplement exhibits a limited effectiveness in the same phages which is perhaps due to a more decisive inhibition, by fluorouracil, of the methylation step leading to the thymine component of deoxyribonucleic acid than of the metabolism of ribonucleic acid. The depression of the production of phage T3, however, appears to concern primarily the "uracil step".

It is not inconceivable that bacteria infected with T2r phage in the presence of fluorouracil and uracil are able to produce some phage protein. Preliminary tests for the presence of phage "ghosts"[19] in lysates of such cells have failed to provide evidence of the formation of killing particles. We are, however, continuing these experiments, including the use of immunological tests for the production of phage protein.

ACKNOWLEDGEMENTS

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REFERENCES

[1] E. Chargaff, *Chemical Aspects of Biological Specificity, The Jesup Lectures* 1959, Columbia University Press, in preparation.
[2] E. Chargaff, in *The Origin of Life on the Earth.* (I.U.B. Symposium Series, Vol. I), Pergamon Press, London, 1959, p. 297.
[3] E. Chargaff, *Transactions, 4th International Congress of Biochemistry,* Vol. XIV, Pergamon Press, London, 1959, p. 21.
[4] A. Lombard and E. Chargaff, *Biochim. Biophys. Acta,* 25 (1957) 549.
[5] J. Horowitz, J. J. Saukkonen and E. Chargaff, *Biochim. Biophys. Acta,* 29 (1958) 222.
[6] J. Horowitz, A. Lombard and E. Chargaff, *J. Biol. Chem.,* 233 (1958) 1517.
[7] C. Heidelberger, N. K. Chaudhuri, P. Danneberg, D. Mooren, L. Griesbach, R. Duschinsky, R. J. Schnitzer, E. Pleven and J. Scheiner, *Nature,* 179 (1957) 663.
[8] J. J. Saukkonen, F. Goodman and E. Chargaff, *Biochim. Biophys. Acta,* 44 (1960) 469.
[9] J. Horowitz, J. J. Saukkonen and E. Chargaff, in preparation.
[10] J. Horowitz and E. Chargaff, *Nature,* 184 (1959) 1213.
[11] G. R. Wyatt and S. S. Cohen, *Nature,* 170 (1952) 1072.
[12] M. H. Adams, *Bacteriophages,* Interscience Publishers, Inc., New York, 1959, p. 443.
[13] B. D. Davis and E. S. Mingioli, *J. Bacteriol.,* 60 (1950) 17.
[14] A. H. Doermann, *J. Gen. Physiol.,* 35 (1952) 645.
[15] J. I. Tomizawa and S. Sunakawa, *J. Gen. Physiol.,* 39 (1956) 553.
[16] S. S. Cohen, J. G. Flaks, H. D. Barner, M. R. Loeb and J. Lichtenstein, *Proc. Natl. Acad. Sci.,* 44 (1958) 1004.
[17] G. S. Stent, in F. M. Burnet and W. M. Stanley, *The Viruses,* Vol. 2, Academic Press, New York and London, 1959, p. 237.
[18] P. B. Danneberg, B. J. Montag and C. Heidelberger, *Cancer Research,* 18 (1958) 329.
[19] R. M. Herriott and J. L. Barlow, *J. Gen. Physiol.,* 41 (1957) 307.

PATTERNS OF CELLULAR CONTROLS OPERATING IN BACTERIOPHAGE REPRODUCTION

II. EFFECT OF 5-FLUOROURACIL ON METABOLIC EVENTS IN BACTERIA INFECTED WITH COLIPHAGE T2r⁺

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SUMMARY

The formation of protein and deoxyribonucleic acid in *E. coli* cells infected with $T2r^+$ phage in the presence of 5-fluorouracil without or with the addition of pyrimidine supplements (uracil, thymidine) was studied. The fluoro compound completely inhibited the synthesis of deoxyribonucleic acid in infected cells; protein still was produced, though at a diminished rate. In the presence of this inhibitor, uracil stimulated only the synthesis of protein, thymidine also that of deoxyribonucleic acid; with both supplements figures surpassing those of control preparations were recorded.

The purine and pyrimidine composition of the deoxyribonucleic acid present in the various systems was determined and the fate of the nucleic acid of the infecting phage was followed through the use of ^{14}C-labeled phage and the estimation of total and specific radioactivity of the nucleic acid components.

INTRODUCTION

The effects of 5-fluorouracil on the multiplication of even- and odd-numbered T phages were studied in the preceding paper[1]. In the present communication an attempt is made to gain some insight into the biochemical phenomena underlying the inhibition caused by the fluoro pyrimidine. The formation of protein and nucleic acids in *E. coli* cells infected with phage $T2r^+$ was followed under conditions simulating those observed in the preceding study and the composition of the deoxyribonucleic acid remaining or synthesized in these systems was determined. In some of the experiments ^{14}C-labeled phage was employed.

EXPERIMENTAL

Materials

The pyrimidines employed have been mentioned before[1]. The purity of all preparations was checked spectrophotometrically and by means of paperchromato-

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graphy in several solvent systems. In the case of 5-fluorouracil use was also made of chromatography on an anion exchange column (Dowex-2, formate; gradient elution with 0.2 M ammonia–0.2 M ammonium formate) which permits a good separation of fluorouracil and uracil.

Strains and media

The bacterial strain used was *E. coli*, strain B. In all experiments phage T2r$^+$ was employed which was obtained through the courtesy of Prof. F. J. RYAN. The synthetic medium and the general methodology were mentioned in the preceding paper[1].

Radioactive phage was prepared by growing the bacteria in the presence of [^{14}C]-glucose (255,000 counts/min/μmole; 6.5 μmoles/ml of culture) followed by infection with a low multiplicity of phage. Marked lysis occurred within 4 to 5 h, when the phage preparation was concentrated and purified by several cycles of alternating centrifugations at low and high speeds[2]. The purified phage had a titer of $8.4 \cdot 10^{11}$/ml and gave 35,600 counts/min/ml.

Experimental arrangements

The bacterial cells were grown in the synthetic medium with aeration at 37°. When a density of about $2 \cdot 10^8$ cells/ml was reached, the culture was divided into portions to which the various supplements, as specified later, were added and the incubation at 37° was continued for 30 min. Infection was then carried out with T2r$^+$ at a ratio of 10 to 15 phage per bacterium. The samples removed at intervals for various determinations were chilled, brought to a 5 % concentration with respect to trichloroacetic acid by the addition of a 50 % solution, and kept at 4° for 1 h.

For the estimation of total protein and nucleic acids the precipitates were collected by centrifugation and washed once with cold 5 % trichloroacetic acid, twice with ethanol–ether (1:3, v/v) and once with ether[3]. The residues were dissolved in 0.2 M sodium hydroxide and kept at 30° for 18 h. Samples of the centrifuged solutions served for the estimation of the nucleic acids and of protein.

For the study of the composition of the deoxyribonucleic acid encountered under various conditions the arrangement of HERSHEY *et al.*[4] was followed. Bovine serum albumin was added to the infected cultures to a final concentration of 0.3 % before precipitation with cold trichloroacetic acid.

In experiments designed to detect the synthesis of small amounts of phage precursor deoxyribonucleic acid, the cells were grown to a density of 3 to $4 \cdot 10^8$/ml and the culture was divided into portions which, after receiving the various pyrimidine supplements, were kept at 37° for 30 min. The samples then were centrifuged and the sediments suspended in the adsorption medium[2] (one-quarter of the original volume) containing the same concentrations of pyrimidine supplements. After the addition of ^{14}C-labeled phage in a ratio of 15 phage per bacterium, the suspensions were kept for 10 min at 37° and the cells were collected by centrifugation and suspended in the growth medium (about three-quarters of the original volume) again containing the pyrimidines employed in the particular experiment. Samples removed at the beginning of the experiment and 3 h later served for the study of deoxyribonucleic acid composition. The individual purines and pyrimidines, separated by paper chromatography, were eluted with 0.01 N HCl; the extracts were, after spectrophotometry, neutralized with NaOH, transferred quantitatively to stainless steel planchets, evaporated to dryness, and their radioactivity was determined.

Analytical procedures

The bacterial and phage assays were mentioned in the preceding paper[1]. Deoxyribonucleic acid was estimated by the DISCHE procedure[5] with a preparation from calf thymus as the reference standard, ribonucleic acid by means of the orcinol reaction[6], an ox liver preparation serving as the standard. The modification of the biuret reaction for the estimation of protein[7] employed bovine serum albumin as the standard. Phosphorus was determined colorimetrically[8]. The radioactive specimens were counted at infinite thinness in a Nuclear-Chicago D47 gas flow counter equipped with a Micromil end window.

RESULTS AND DISCUSSION

Changes in nucleic acid and protein contents

The global effects of the presence of 5-fluorouracil in the growth medium on the synthesis of deoxyribonucleic acid and protein are shown in Fig. 1. The fluoro compound completely inhibits the formation of deoxyribonucleic acid by cells infected with $T2r^+$ phage during the observation period of 4 h. This block is not abolished by supplementation with uracil; but thymidine permits a four-fold increase in this nucleic acid: an increase which is, however, significantly lower than in the untreated culture. The addition of both uracil and thymidine to the system containing fluorouracil results in the restoration of the capacity to make deoxyribonucleic acid in amounts that, in fact, often surpass those formed under comparable conditions in untreated phage-bearing cultures. Whether the slight initial rise observed even under conditions of inhibition (Sections II and III of Fig. 1) is meaningful cannot be said.

Protein is accumulated in the cells treated with 5-fluorouracil at a much slower rate than in the absence of the inhibitor, the total amounting to only about one-half of that formed by the untreated cells. The presence of thymidine improves the yield slightly, that of uracil appears to increase both rate of synthesis and total amount considerably. When both supplements are added, a complete reversal of the inhibition, or even an increase over the control cultures, is again recorded.

The observation that thymidine as the only supplement of fluorouracil is able to stimulate the synthesis of considerable amounts of phage deoxyribonucleic acid (Section IV of Fig. 1 and Table I to be discussed below) and even of phage particles[1] could lead to the surmise that the administration of thymidine made some uracil available to the cellular system. We are, however, not aware of any evidence of such a demethylation reaction, except for a recent, equally indirect, suggestion with respect to *Bacillus cereus*[9]. That such a conversion, if it occur indeed, cannot be massive is demonstrated by the inability of thymidine to replace uracil effectively in the uracil-deficient *E. coli* mutant 63–86 (see ref. 10, 11). (Unpublished observations by Dr. J. HOROWITZ.) It should also be remembered that, as we have shown in the preceding paper[1], thymidine is unable to replace uracil as a requisite for the multiplication of T3 phage in cells treated with fluorouracil.

We have omitted, from Fig. 1, observations on changes in ribonucleic acid content under the conditions of this experiment. It is generally stated that the synthesis of this component ceases immediately upon infection of the bacterium by phage[12], although a metabolically active species of ribonucleic acid[13], differing in composition from the ribonucleic acid of the host cell[14], has been observed in infected cells. (Compare the interesting discussion of this problem on p. 56 of ref. 15.) We noticed occasionally slight increases in ribonucleic acid in the initial stages of infection (within 60 min), especially in the systems containing, in addition to fluorouracil, either uracil alone or uracil and thymidine.

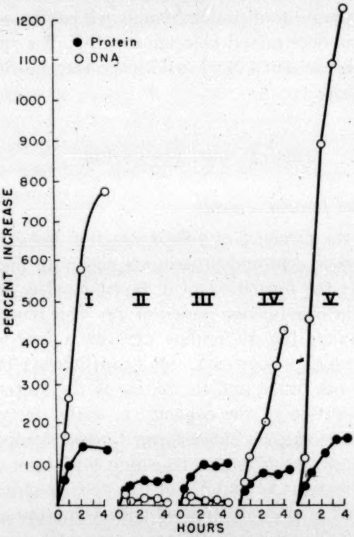

Fig. 1. Formation of protein and deoxyribonucleic acid in *E. coli*, strain B, infected with T2r$^+$ phage in the presence of various pyrimidines (5-fluorouracil, 50 μg/ml; uracil, 20 μg/ml; thymidine, 50 μg/ml). See text for experimental conditions. The addition of phage occurred at zero time. The systems represented are: I, no additions; II, fluorouracil; III, fluorouracil and uracil; IV, fluorouracil and thymidine; V, fluorouracil, uracil and thymidine.

It should be mentioned that, under the experimental conditions followed here, approx. 99 % of the cells were infected within 10 min after phage addition.

The findings on T2r$^+$ described here may be correlated with the results on the production of T2r phage particles summarized in Table III of the preceding communication[1]. In that table it was shown that under conditions of high phage input only about 0.5 % of the normal phage yield was obtained in the presence of 5-fluorouracil; that this figure did not change materially when uracil was added; but that the supplementation of the fluoro compound with thymidine raised the yield to about one-fifth of the normal. These observations are in good agreement with the trend in the formation of deoxyribonucleic acid under the various conditions studied in the present paper. A rise in protein, on the other hand (compare Section III of Fig. 1), is not in itself indicative of the ability to produce phage particles.

TABLE I

COMPOSITION OF DEOXYRIBONUCLEIC ACID IN *E. coli* CELLS INFECTED WITH T2r$^+$ IN PRESENCE OF 5-FLUOROURACIL AND PYRIMIDINE SUPPLEMENTS

The values for composition are reported as µmoles/100 ml culture, with the exception of the parenthetical figures which indicate the particular constituent in mole % of total bases recovered. Abbreviations: Ø, phage T2r$^+$; FU, 5-fluorouracil (50 µg/ml); U, uracil (20 µg/ml); dTR, thymidine (50 µg/ml). The times indicate hours after infection.

Experiment No. *Conditions of experiments*	*I* *At start, no additions*	*II* *Ø; 3 h*	*III* *Ø, FU; 3 h*	*IV* *Ø, FU, U; 3 h*	*V* *Ø, FU, dTR; 3 h*	*VI* *Ø, FU, U, dTR; 3 h*
"DNA-P" in total preparation*	0.94	16.32	0.91	0.98	4.64	13.73
DNA-P in extract*	0.73	10.80	0.51	0.56	3.36	10.54
Sum of recovered purines and pyrimidines	0.73	9.22	0.56	0.60	3.47	9.07
Composition of DNA						
Adenine	0.18 (25)	2.80 (30)	0.16 (29)	0.17 (28)	1.04 (30)	2.81 (31)
Guanine	0.18 (25)	1.83 (20)	0.10 (18)	0.11 (18)	(0.60 17)	1.50 (17)
Cytosine	0.17 (23)	0.03	0.06 (11)	0.05 (8)	0.10 (3)	0.08 (1)
5-Hydroxymethylcytosine		1.62 (18)	0.06 (11)	0.07 (12)	0.51 (15)	1.49 (16)
Thymine	0.20 (27)	2.94 (32)	0.18 (32)	0.20 (33)	1.22 (35)	3.19 (35)
Molar proportions						
Purines to pyrimidines	1.0	1.0	0.9	0.9	0.9	0.9
Adenine to thymine	0.9	1.0	0.9	0.9	0.9	0.9
Guanine to cytosine + hydroxymethylcytosine	1.1	1.1	0.8	0.9	1.0	1.0

* Determined by means of the diphenylamine reaction.

TABLE II

COMPOSITION OF DEOXYRIBONUCLEIC ACID AND DISTRIBUTION OF RADIOACTIVITY IN *E. coli* CELLS INFECTED WITH ^{14}C-LABELED T2r$^+$ IN PRESENCE OF 5-FLUOROURACIL AND URACIL

The values describing the distribution of individual purines and pyrimidines are reported in μmoles/100 ml culture (with the particular constituent as mole % of total bases recovered in parentheses). The total radioactivity refers to counts/min/100 ml culture (with the specific radioactivities given in brackets as counts/min/μmole). For conditions of experiments see Table I.

Experiment No.	*VII*		*VIII*		*IX*		*X*	
Conditions of experiments	*Ø, FU, 0 h*		*Ø, FU, 3 h*		*Ø, FU, F, 0 h*		*Ø, FU, U, 3 h*	
	Composition	*Total radioactivity*	*Composition*	*Total radioactivity*	*Composition*	*Total radioactivity*	*Composition*	*Total radioactivity*
Sum of recovered purines and pyrimidines	1.40	568 [410]	0.68	380 [560]	1.53	606 [400]	0.96	500 [520]
Adenine	0.39 (28)	177 [450]	0.19 (28)	95 [500]	0.42 (27)	192 [460]	0.26 (27)	135 [520]
Guanine	0.30 (21)	94 [310]	0.14 (21)	78 [560]	0.33 (22)	91 [280]	0.19 (20)	69 [360]
Cytosine	0.28 (20)	8	0.09 (13)	6	0.31 (20)	10	0.16 (17)	12
5-Hydroxymethylcytosine	0.04 (3)	89 [2230]	0.06 (9)	56 [930]	0.05 (3)	94 [1880]	0.06 (6)	91 [1520]
Thymine	0.39 (28)	200 [510]	0.20 (29)	145 [730]	0.42 (27)	219 [520]	0.29 (30)	193 [670]
			Molar proportions					
Purines to pyrimidines	1.0		0.9		1.0		0.9	
Adenine to thymine	1.0		1.0		1.0		0.9	
Guanine to cytosine + hydroxymethylcytosine	0.9		0.9		0.9		0.9	
Adenine + thymine to guanine + cytosine + hydroxymethylcytosine	1.3		1.3		1.2		1.3	
Proportion of phage DNA in total DNA, %*	16		50		16		34	

* The figures for hydroxymethylcytosine served for the calculation of the percentage of phage DNA, with the assumption that the latter contained 32 mole % each of adenine and thymine and 18 mole % each of guanine and hydroxymethylcytosine.

Composition of deoxyribonucleic acid

The nature of the deoxyribonucleic acid found in the bacterial cells under various conditions was studied by analyzing the base composition of the preparations; the results are summarized in Table I. The findings on the nucleic acid composition in untreated bacteria (Expt. I) are in good agreement with previous work in which the same, relatively crude analytical method was used[4], as well as with observations on the composition of the intact deoxyribonucleic acid of different *E. coli* strains[16,17]. The recovery of purines and pyrimidines was very satisfactory when compared with the figures for the nucleic acid contents of the extracts (based on the determination of deoxy sugar). Much less satisfactory was the agreement between the deoxy sugar values of the total preparations and the extracts, particularly in the experiments (Nos. III and IV) employing fluorouracil alone or together with uracil.

The procedures used in the present study seem to have a tendency to yield too high values for thymine, but even so the characteristic, nearly equimolar base distribution in the deoxyribonucleic acid of *E. coli* is apparent in the results of Expt. I. After 3 h, in the untreated infected cells (Expt. II) about 17 times as much nucleic acid is found as in noninfected bacteria at the start, but this nucleic acid now has nearly the composition of that of phage[18], and the almost complete disappearance of polymer-bound cytosine demonstrates the destruction of the bacterial nucleic acid during this period. In the systems infected with phage in the presence of 5-fluorouracil and supplemented with both uracil and thymidine or only with the latter (Expts. V and VI) 85 to 90 % of the deoxyribonucleic acid shows the characteristics of phage nucleic acid although, as expected, the absolute amount of nucleic acid formed is much greater in the first instance. The decrease in the cytosine content of these preparations was somewhat smaller in Expt. II; and this may mean that the breakdown of host nucleic acid did not quite go so far in the inhibited systems.

Under conditions of infection in the presence of fluorouracil alone or together with uracil (Expts. III and IV) no increase in deoxyribonucleic acid beyond the control value is recorded; and in these systems both cytosine and hydroxymethylcytosine, in almost equal quantities, are found*. From the figure for the latter pyrimidine it can be calculated that more than one-half of the total deoxyribonucleic acid consisted of phage nucleic acid. There must, consequently, have occurred considerable degradation of bacterial nucleic acid under these circumstances.

It appeared of some interest to reinvestigate the stages in which no net synthesis of deoxyribonucleic acid was apparent, *i.e.* in cells infected with phage in the presence of fluorouracil alone or together with uracil, by a different technique permitting a more direct decision about the fate of the components of the infecting phage. In this series of studies ^{14}C-labeled phage was employed and special care was taken to remove excess phage, as described above. The results are shown in Table II. We shall first consider the findings on the composition of the total nucleic acid of the preparations and then those relating to the fate of the infecting phage nucleic acid. Expts. VII and IX describe the state of the internal deoxyribonucleic acid immediately after the uptake of phage in the presence of fluorouracil either alone or together with uracil. On the basis of the hydroxymethylcytosine content, about one-sixth of the total nucleic acid is attributable to the phage. This proportion increases considerably after 3 h (Expts. VIII and X) which could, superficially, be taken to mean that more bacterial than phage nucleic acid is destroyed during this period; it could, however, be simply due to a more complete extraction of the latter in these preparations. But the content in deoxyribonucleic acid unquestionably is much lower after 3 h. The figures for hydroxymethylcytosine can also be used to compute the composition of that portion of the total nucleic acid that is attributable to the host; and when this is done, proportions are found that are not too far from those expected for *E. coli* nucleic acid. We have also included in this table some of the characteristic molar proportions of the nucleic acid which, considering the crudeness of the methods, are not unsatisfactory.

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* A minor component, probably identical with the one mentioned by HERSHEY *et al.*[4], was frequently seen on the chromatograms of the hydrolysates of these preparations. It moved in propanol–HCl together with adenine, in propanol–NH$_3$ with cytosine.

Turning now to the findings on radioactivity we notice first in Table II that some of the total radioactivity is lost in the course of 3 h: about 33 % when going from Expt. VII to VIII; 17 % when Expts. IX and X are compared. This drop is, however, inferior to that shown by the balances of total recovered purines and pyrimidines which amounts to 51 and 37 %, respectively, in the two sets of experiments. This may again be taken to signify that relatively more bacterial than phage nucleic acid has disappeared during the experimental period. With one exception, the specific activities of the individual components rose during the interval of 3 h. Only the specific activity of hydroxymethylcytosine decreased; and this, could, perhaps, indicate that some synthesis of this pyrimidine took place even in the presence of the inhibitor: a conclusion to be treated with caution in view of the low concentration of this component. Some differences between cells treated with fluorouracil alone and those supplemented also with uracil are noticeable, but need not be discussed in detail.

The observation that synthesis of some 5-hydroxymethylcytosine may occur even in the presence of 5-fluorouracil (Table II) and the effect of thymidine in permitting a limited resumption of the synthesis of phage precursors and of phage could, perhaps, be reconciled by the assumption that fluorouracil did not completely block the formation of "deoxycytidylate hydroxymethylase" and "thymidylate synthetase"[19, 20], but only the action of the latter enzyme.

It may be of some interest to tabulate the percentage contribution of the individual nucleic acid constituents to the total radioactivity observed in the various

TABLE III

DISTRIBUTION OF TOTAL RADIOACTIVITY AMONG DEOXYRIBONUCLEIC ACID CONSTITUENTS IN *E. coli* CELLS INFECTED WITH ^{14}C-LABELED T2r$^+$ IN PRESENCE OF 5-FLUOROURACIL AND URACIL

Compare Table II for details. For the computation, the counts of cytosine were deducted from the total radioactivities listed in Table II.

DNA constituent	*% of total radioactivity*			
	Experiment No.			
	VII	*VIII*	*IX*	*X*
Adenine	32	25	32	28
Guanine	17	21	15	14
5-Hydroxymethylcytosine	16	15	16	19
Thymine	36	39	37	40

experiments; and such a comparison is provided in Table III. In the samples taken immediately after infection, *i.e.*, in Expts. VII and IX, essentially the proportions characteristic for T2r$^+$ phage nucleic acid[18] are observed, though here again (compare the discussion of Table I) the contribution of thymine is somewhat high. After 3 h (Expts. VIII and X) the radioactivity patterns become severely distorted; but it cannot be decided whether the apparently greater conservation of pyrimidine nucleotides is meaningful. In any event, it may be conjectured that the phage nucleic acid is not preserved intact during this period.

ACKNOWLEDGEMENTS

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REFERENCES

[1] F. GOODMAN, J. J. SAUKKONEN AND E. CHARGAFF, *Biochim. Biophys. Acta*, 44 (1960) 458.
[2] A. D. HERSHEY AND M. CHASE, *J. Gen. Physiol.*, 36 (1952) 39.
[3] D. ELSON, T. GUSTAFSON AND E. CHARGAFF, *J. Biol. Chem.*, 209 (1954) 285.
[4] A. D. HERSHEY, J. DIXON AND M. CHASE, *J. Gen. Physiol.*, 36 (1953) 777.
[5] K. BURTON, *Biochem. J.*, 62 (1956) 315.
[6] W. MEJBAUM, *Z. physiol. Chem., Hoppe-Seyler's*, 258 (1939) 117.
[7] C. F. CRAMPTON, R. LIPSHITZ AND E. CHARGAFF, *J. Biol. Chem.*, 206 (1954) 499.
[8] E. J. KING, *Biochem. J.*, 26 (1932) 292.
[9] B. H. SELLS, *Biochem. Pharmacol.*, 2 (1959) 255.
[10] J. HOROWITZ, J. J. SAUKKONEN AND E. CHARGAFF, *Biochim. Biophys. Acta*, 29 (1958) 222.
[11] J. HOROWITZ AND E. CHARGAFF, *Nature*, 184 (1959) 1213.
[12] S. S. COHEN, *Advances in Virus Research*, 3 (1955) 1.
[13] A. D. HERSHEY, *J. Gen. Physiol.*, 37 (1953) 1.
[14] E. VOLKIN AND L. ASTRACHAN, in W. D. MCELROY AND B. GLASS, *The Chemical Basis of Heredity*, Johns Hopkins Press, Baltimore, 1957, p. 686.
[15] A. D. HERSHEY, *Advances in Virus Research*, 4 (1957) 25.
[16] E. CHARGAFF, H. M. SCHULMAN AND H. S. SHAPIRO, *Nature*, 180 (1957) 851.
[17] A. N. BELOZERSKY AND A. S. SPIRIN, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. III, Academic Press Inc., New York, in the press.
[18] G. R. WYATT AND S. S. COHEN, *Biochem. J.*, 55 (1953) 774.
[19] S. S. COHEN, J. G. FLAKS, H. D. BARNER, M. R. LOEB AND J. LICHTENSTEIN, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 1004.
[20] J. G. FLAKS AND S. S. COHEN, *J. Biol. Chem.*, 234 (1959) 2981.

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STUDIES OF DEOXYRIBONUCLEIC ACID SYNTHESIS AND CELL GROWTH IN THE DEOXYRIBOSIDE-REQUIRING BACTERIA, *LACTOBACILLUS ACIDOPHILUS*

III. IDENTIFICATION OF THYMIDINE DIPHOSPHATE RHAMNOSE

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SUMMARY

A new deoxynucleotide, thymidine diphosphate rhamnose, was isolated from *Lactobacillus acidophilus* R-26 which had been subjected to a treatment which induced an accumulation of deoxyribosidic compounds. The results presented indicate that in the nucleotide, thymidine-5′-monophosphate is linked to rhamnose-1-phosphate through a pyrophosphate bond. The possible metabolic function of the nucleotide is discussed.

INTRODUCTION

In previous papers[1,2], we have presented evidence for the occurrence of a new thymidine nucleotide in the acid-soluble extract of *Lactobacillus acidophilus* R-26. It has also been reported that the same nucleotide accumulates markedly when DNA synthesis is induced in the resting cells of this micro-organism by replenishment with thymidine after depletion of uracil and deoxyriboside[3]. The nucleotide has now been isolated from these cells and identified as thymidine diphosphate rhamnose (TDP-rhamnose).

Preliminary reports of this work have been published[4,5].

MATERIALS AND METHODS

The micro-organism and culture medium

L. acidophilus strain R-26 was grown in the synthetic medium, the composition of which has been given previously[3]. The procedures for the depletion and replenishment were shown in the legend of Fig. 2. The cells were harvested by centrifugation in the cold, washed in saline and frozen with CO_2–ethanol.

Abbreviations: DNA, deoxyribonucleic acid; TMP, TDP and TTP, thymidine 5′-mono-, di- and triphosphate; UDP, uridine 5′-diphosphate; GDP, guanosine 5′-diphosphate; CDP, cytidine 5′-diphosphate; AMP, adenosine 5′-monophosphate; DPN, diphosphopyridine nucleotide; PCA, perchloric acid; TCA, trichloroacetic acid.

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Preparation of the acid-soluble extract

The frozen cells were thawed, extracted with ice-cold 0.5 *N* PCA and washed twice with 0.2 *N* PCA. The original extract and the washings were combined, neutralized with KOH and concentrated by lyophilization, the precipitate of $KClO_4$ being removed by centrifugation.

Ion-exchange chromatography

The acid-soluble extract was added onto a column of Dowex-1-formate (X 10, 200–400 mesh), 1.25 × 16 cm, and fractionated as below by using a modification of the extended gradient-elution chromatographic method with the "formic acid system"[6].

After washing the column with 35 ml of water, elution was carried out in the cold by introducing successively the following eluents into a mixing flask initially filled with 500 ml of water: 100 ml of 1 *N* formic acid; 500 ml of 4 *N* formic acid; 500 ml of 0.2 *M* ammonium formate in 4 *N* formic acid; 300 ml of 0.4 *M* ammonium formate in 4 *N* formic acid; 650 ml of 1 *M* ammonium formate in 4 *N* formic acid. The flow rate was 15–25 ml/h, and samples containing 5 ml effluent were collected. The extinction at 260 mμ and 280 mμ was measured for each tube in a Beckman DU spectrophotometer. Effluent fractions corresponding to the peaks or portions of peaks were pooled and lyophilized to remove formic acid and ammonium formate.

Chromatography of the phosphodiesterase digest of the isolated nucleotide was performed with a smaller column (0.6 × 17 cm) and a 100 ml mixing flask.

Paper chromatography

Descending paper chromatography was carried out on Whatman No. 1 paper in the following solvents: Isobutyric acid–1 *N* NH_4OH (10:6) (modified from KREBS AND HEMS[7]); butanol saturated with water[8]; butanol–acetic acid–water (4:1:5)[8]; butanol–ethanol–water (104:62:34)[9]; phenol saturated with water[8]; ethyl acetate–pyridine–water (8:2:1)[10]; ethyl acetate–acetic acid–water (3:1:3)[11]; butanol–pyridine–water (6:4:3)[12].

Nucleotides were located on the chromatograms by means of u.v. photography with a filter (253.7 mμ) of the Scientific Research Institute*. For preparative purposes, the nucleotide was chromatographed as a band and eluted from the paper with water.

Sugars were detected by spraying with aniline–hydrogen phthalate reagent[13]. For the isolation of sugar liberated on acid hydrolysis from the nucleotide, the hydrolyzed nucleotide was run in butanol–ethanol–water as a band. After chromatography a guide strip was cut out from the paper and sprayed with aniline–hydrogen phthalate. The sugar was located by reference to this guide strip and eluted with water.

Paper electrophoresis

The paper electrophoresis of sugars was carried out with the apparatus described by MARKHAM AND SMITH[14]. Samples were run in 0.05 *M* borate buffer, pH 8.6[15], on Whatman No. 1 for 1.5 h at a potential gradient of about 30 Volts/cm. Sugars were made visible by spraying with aniline–hydrogen phthalate acidified with acetic acid.

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* Kindly supplied by Dr. E. IWASE.

Charcoal treatment of the nucleotide

The nucleotide sample eluted from the paper chromatogram was further purified by charcoal treatment. Norit A which had been successively washed with 50 % ethanol containing 1 % conc. ammonia, 0.1 *N* HCl and water was added to the nucleotide solution adjusted to pH about 5. After vigorous stirring for 30 min the charcoal was precipitated by centrifugation. It was then re-suspended in cold 2 *N* formic acid, centrifuged and washed with water until the washings were neutral[16]. The nucleotide was eluted from the charcoal with cold 50 % ethanol containing 0.7 % conc. ammonia. The ammoniacal solution of nucleotide obtained was evaporated *in vacuo* and taken up in a small volume of water. In some cases the solution of the nucleotide in 0.1 *N* HCl was passed through a small column of Dowex-50-H^+ to ensure complete removal of ammonium ions.

Analyses

Phosphorus determination was carried out according to MARTIN AND DOTY[17]. Total phosphorus was determined after digestion with PCA. Determination of labile phosphorus was carried out by a 10 min hydrolysis in 1 *N* HCl at 100°. Nitrogen was determined according to LEVY AND PALMER[18]. Reducing sugar was estimated by the method of PARK AND JOHNSON[19]; methylpentose (6-deoxyhexose) by the method of DISCHE AND SHETTLES[20].

Deoxyriboside was assayed microbiologically after treatment of the sample with snake venom[1].

Biochemicals

Thymidine and 2′,3′-cytidylic acid were purchased from Schwarz Laboratories, Inc.; TMP from the California Foundation for Biochemical Research; L-rhamnose from Wako Pure Chemical Industries, Ltd.; L-fucose from Bios Laboratories, Inc. TDP and TTP were obtained through the courtesy of Professor A. KORNBERG, Stanford University. Purified snake (*Agkistrodon blomhoffii*) venom phosphodiesterase and bull semen 5′-nucleotidase were generously provided by Dr. S. IWANAGA, Kyoto University. Crude snake venom, *Agkistrodon blomhoffii* was obtained from Bunkyu Do Co. (Tokyo).

RESULTS

Isolation of the nucleotide

In Fig. 1 the ion-exchange chromatogram of an acid-soluble extract prepared from a growing culture in the complete medium was shown, together with an example of the behavior of authentic TMP, TDP and TTP in the same system. The major portion of the deoxyribosidic compounds in the original extract was recovered in a fraction eluted in tubes No. 180–200. It is evident that the behavior of the chief deoxyribosidic compound in the extract, which was later identified as TDP-rhamnose, was distinct from any known thymidine nucleotide.

As reported in an earlier paper[3], DNA synthesis can be induced in resting cells by adding thymidine to the bacteria previously starved of uracil and deoxyriboside. Under these conditions a marked accumulation of acid-soluble deoxyribosidic compounds precedes the active synthesis of DNA. Acid-soluble extracts were prepared

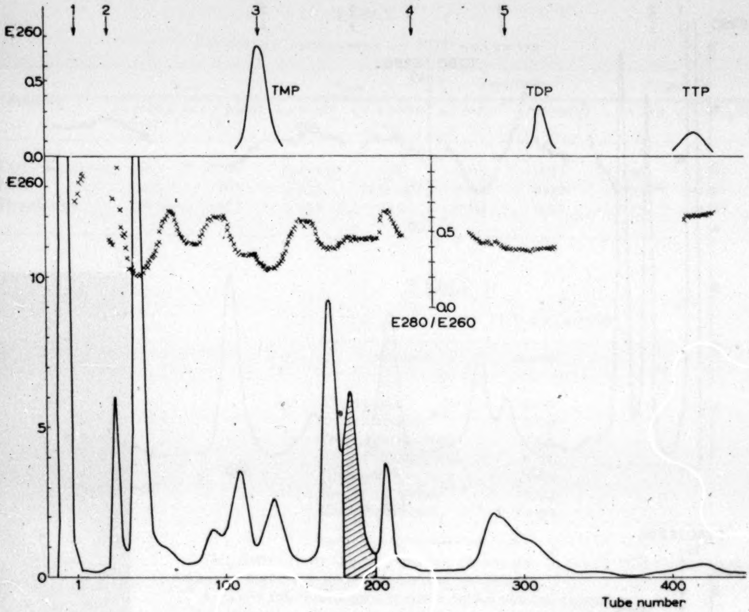


Fig. 1. Ion-exchange chromatogram of the acid-soluble extract of normally grown cells. The shaded area indicates the fraction containing chief deoxyribosidic compound (TDP-rhamnose). In the upper portion of the figure the behavior of authentic thymidine nucleotides is shown. The extract was prepared from 35 l culture grown in a complete medium containing thymidine and added onto a column of Dowex-1-formate (X 10, 200–400 mesh) 1.25 × 16 cm. Extended gradient elution was carried out by introducing the following eluents at the indicated points into the reservoir which was connected to a 500 ml mixing flask initially filled with water: (1) 100 ml of 1 *N* formic acid; (2) 500 ml of 4 *N* formic acid; (3) 500 ml of 0.2 *M* ammonium formate in 4 *N* formic acid; (4) 300 ml of 0.4 *M* ammonium formate in 4 *N* formic acid; (5) 650 ml of 1 *M* ammonium formate in 4 *N* formic acid.

from the cells subjected to such treatments and chromatographed on Dowex-1 columns (*cf.* Figs. 2a and 2b).

A large amount of deoxyribosidic compound (55–75 % of the total deoxyribosidic compounds in the extract) was eluted in approximately the same region (tubes No. 186–214 in Expt. 1; No. 181–204 in Expt. 2) as in the chromatogram of the extract of normal cells. However, it will be noticed that the ratio, E280/E260 of this fraction was much higher than that of the corresponding fraction of the extract from normal cells (0.45–0.47 in the normal cells; 0.55–0.60 in the treated cells). As this fraction contained a uridine nucleotide (E280/E260 = 0.38) in addition to the thymidine nucleotide under consideration (E280/E260 = 0.73), the higher value of the ratio E280/E260 of the fraction indicated the relatively high proportion of the thymidine nucleotide.

The crude fraction so prepared was subjected, after removal of formic acid and ammonium formate by lyophilization, to paper chromatography in isobutyric acid–

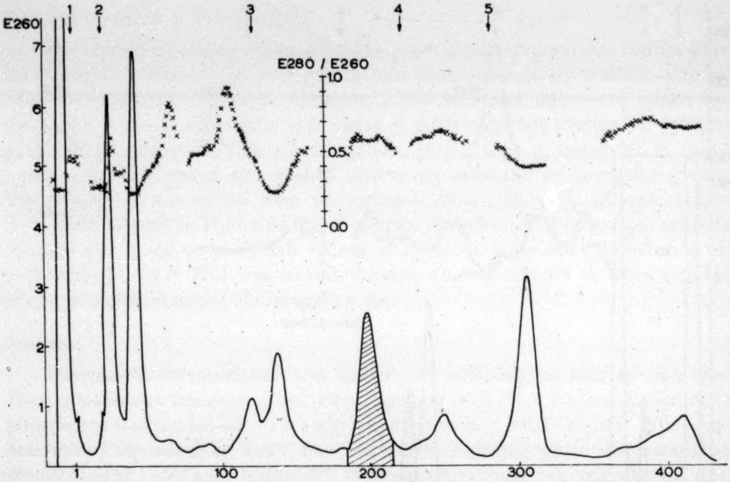


Fig. 2a.

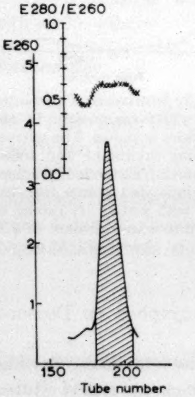


Fig. 2b.

Fig. 2a and 2b. Ion-exchange chromatogram of the acid-soluble extract of the cells subjected to treatment inducing the accumulation of deoxyribosidic compounds (a: Expt. 1; b: Expt. 2). The shaded area indicates the fraction containing chief deoxyribosidic compound (TDP-rhamnose). Cells grown overnight in the complete medium were washed and re-suspended at 0.1 the original cell concentration in 15 l (Expt. 1) or 18 l (Expt. 2) of the medium deficient in uracil and deoxyriboside. After 5.5 h incubation in this medium at 37°, cells were supplied with excess (40 μmoles/l) thymidine, and incubated further for 30 min. The acid-soluble extract was prepared from these cells and chromatographed in the same manner as in Fig. 1.

NH_4OH. Two u.v.-absorbing components ($R_F = 0.35$ and 0.20) were separated on the chromatogram (Fig. 3). On examination of each component after elution from the paper, the fast component was found to have the spectrum of thymidine and to show deoxyribosidic growth activity after venom treatment[1], while the slow component having the spectrum of uridine was microbiologically inactive.

The fast component was isolated by large scale chromatography, adsorbed on Norit A and eluted from the charcoal with ammoniacal ethanol–water. The ammoniacal solution obtained was evaporated *in vacuo*.

TABLE I

SPECTRAL PROPERTIES OF TDP-RHAMNOSE

Substance	*λ_{max}*		*λ_{min}*		*E250/E260*		*E280/E260*	
	0.1 N HCL (mμ)	*0.1 N NaOH (mμ)*	*0.1 N HCl (mμ)*	*0.1 N NaOH (mμ)*	*0.1 N HCl*	*0.1 N NaOH*	*0.1 N HCl*	*0.1 N NaOH*
TDP-rhamnose	267	266	234–235	246	0.65	0.71	0.73	0.68
TMP	267	267	235	245–246	0.63	0.72	0.73	0.68
Thymidine	267	267	235	245	0.63	0.72	0.73	0.68

TABLE II

ANALYTICAL DATA FOR TDP-RHAMNOSE

Substance	*μmoles*
Thymidine*	1.00
Deoxyriboside**	0.99
Total phosphorus	2.05
Labile phosphorus	0.91
Nitrogen	2.05
Reducing value***	0.99
Methylpentose	0.98

* Calculated from the absorbance at 260 mμ in 0.1 *N* HCl by assuming a value of 8,400 for molar extinction coefficient.
** Assayed microbiologically after snake venom digestion.
*** Expressed as rhamnose equivalent.

Fig. 3. Paper chromatogram of the crude fraction of TDP-rhamnose. The fast component is TDP-rhamnose. Solvent: *iso*-butyric acid–1 *N* NH_4OH (10:6).

By these procedures, 7–10 μmoles of the nucleotide were prepared, starting from 15 l culture.

Identification of the nucleotide

General properties: The u.v. absorption spectrum of the isolated nucleotide was in agreement with the spectrum of TMP and thymidine both in acidic and basic solutions (Fig. 4; Table I). Microbiological assay after venom treatment[1] proved the presence of deoxyriboside in the amount expected from u.v. absorption (Table II).

The nucleotide contained two moles organic phosphate per mole thymidine and upon treatment with 1 *N* HCl at 100° for 10 min liberated one mole phosphate in the inorganic form (Table II).

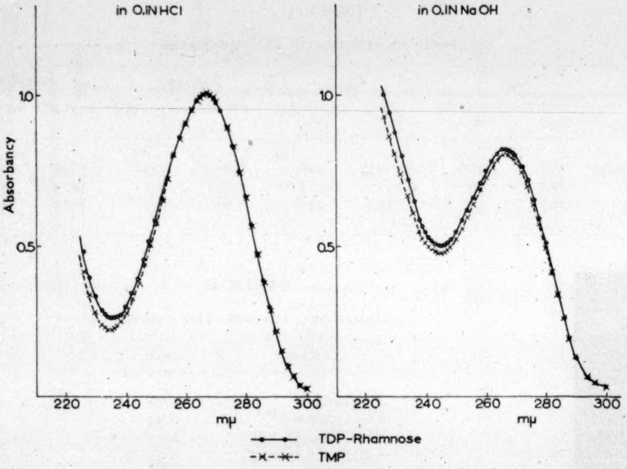


Fig. 4. u.v.-absorption spectra of TDP-rhamnose as compared with the spectra of TMP.

Nitrogen analysis of the sample, which was passed through a column of Dowex-50 for complete removal of contaminating ammonium ions, gave 2 atoms of nitrogen per mole, indicating that it did not contain nitrogen in excess of that accounted for by the thymine moiety.

Mild hydrolysis in 0.01 N HCl at 100° for 10 min yielded a reducing group measurable by ferricyanide reduction[19].

Identification of rhamnose: Various color reactions for sugars were carried out on the hydrolysate of the nucleotide. The following reactions[21] were negative: diphenylamine for 2-deoxysugars; orcinol for pentoses and hexoses; cysteine–carbazole–

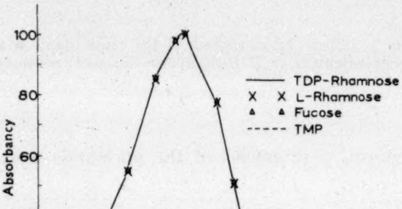


Fig. 5. Absorption spectrum of the reaction product formed in the cysteine–H_2SO_4 reaction for methylpentoses.

H_2SO_4 for ketoses and trioses; carbazole–H_2SO_4 for uronic acids; Elson–Morgan for amino sugars.

The only positive result was obtained with the cysteine–H_2SO_4 reaction for methylpentoses (6-deoxyhexoses)[20]. The nucleotide preparation as well as authentic rhamnose and fucose formed a reaction product with an absorption maximum at 400 mμ. The whole absorption curve of the reaction product formed by the nucleotide was in complete agreement with that of the authentic methylpentoses (Fig. 5). The

TABLE III

R_F VALUES OF THE SUGAR COMPONENT LIBERATED BY MILD ACID HYDROLYSIS OF TDP-RHAMNOSE AND AUTHENTIC METHYLPENTOSES

TDP-rhamnose was hydrolyzed in 0.01 *N* HCl at 100° for 10 min. The sugar in the hydrolysate was isolated by large scale chromatography in ethanol–butanol–water and then subjected to a second paper chromatography using 7 different solvent systems.

Solvents	*Sugar liberated from TDP-rhamnose*	L-*Rhamnose*	L-*Fucose*
Butanol saturated with water	0.24	0.24	0.18
Butanol–acetic acid–water (4:1:5)	0.30	0.31	0.24
Butanol–ethanol–water (104:62:34)	0.49	0.49	0.43
Phenol saturated with water	0.56	0.56	0.64
Ethyl acetate–acetic acid–water (3:1:3)	0.34	0.34	0.28
Ethyl acetate–pyridine–water (8:2:1)	0.34	0.34	0.22
Butanol–pyridine–water (6:4:3)	0.55	0.55	0.46

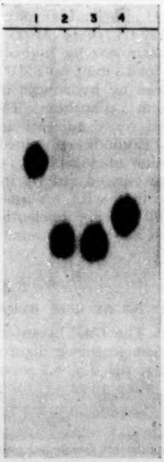


Fig. 6a.

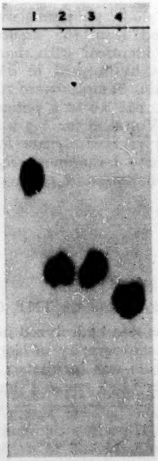


Fig. 6b.

Fig. 6(a) and (b). Paper chromatograms showing that the sugar liberated from the nucleotide (TDP-rhamnose) is identical with rhamnose. (a): Butanol–ethanol–water (104:62:34); (b): Phenol saturated with water. (1) D-glucose; (2) sugar component liberated from TDP-rhamnose; (3) L-rhamnose; (4) L-fucose.

stability of the reaction product to dilution with water[20] was also consistent with the conclusion that the reacting substance in the nucleotide is methylpentose. The amount of methylpentose per mole thymidine was approximately 1 when calculated using rhamnose or fucose as the standard. As shown in Fig. 5, TMP failed almost completely to form a colored product under the conditions of this reaction.

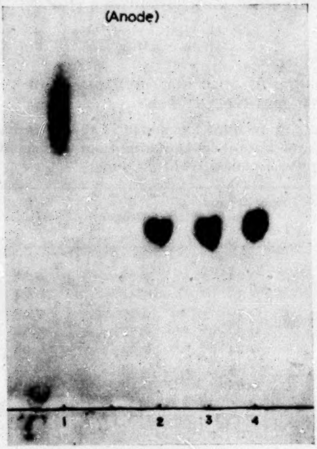


Fig. 7. Paper electrophoresis showing that the sugar liberated from the nucleotide (TDP-rhamnose) is identical with rhamnose. TDP-rhamnose was hydrolyzed in 0.01 N HCl at 100° for 10 min. Samples were run in 0.05 M borate buffer, pH 8.6 at a potential gradient of about 30 Volts/cm for 1.5 h. (1) L-fucose ($R_{gluc.} = 0.65$); (2) Hydrolysate ($R_{gluc.} = 0.40$); (3) hydrolysate + L-rhamnose ($R_{gluc.} = 0.40$); (4) L-rhamnose ($R_{gluc.} = 0.41$).

Fig. 8. Paper chromatography in isobutyric acid–1 N NH_4OH (10:6) of: (1) TDP liberated from TDP-rhamnose by hydrolysis in 0.01 N HCl at 100° for 10 min; (2) TMP liberated from TDP-rhamnose by hydrolysis in 1 N HCl at 100° for 10 min; (3) authentic TMP; (4) thymidine obtained by 5′-nucleotidase digestion of sample 2; (5) thymidine obtained by 5′-nucleotidase digestion of sample 3. 5′-nucleotidase digestion was carried out by incubating 0.11 μmole nucleotide with 0.1 μmole $MgCl_2$ and 4.4 units[22] bull semen 5′-nucleotidase at pH 8–9 at 37° for 30 min.

TABLE IV

IDENTIFICATION OF TMP LIBERATED FROM TDP-RHAMNOSE BY ACID HYDROLYSIS

TDP-rhamnose was hydrolyzed in 1 N HCl at 100° for 10 min. The TMP liberated was isolated by paper chromatography in isobutyric acid–NH_4OH. For 5′-nucleotidase digestion, 0.11–0.15 μmole nucleotide was incubated with 20 μmoles glycine buffer, pH 8.5, 0.1 μmole $MgCl_2$ and 4.4 units[22] bull semen 5′-nucleotidase in a total volume of 0.9 ml at 37° for 30 min.

Samples	*Total P (μmoles per μmole nucleoside)*	*P split by 5′-nucleotidase (μmoles per μmole nucleoside)*
TMP liberated from TDP-rhamnose	1.02	0.95
Authentic TMP	1.04	0.96
2′,3′-Cytidylic acid		0.00

The sugar was isolated from the acid hydrolysate by large scale chromatography in butanol–ethanol–water, and run with authentic sugars in 7 different solvent systems. As is evident from Table III and Fig. 6 the mobility of the sugar in the hydrolysate was identical with the mobility of rhamnose in all 7 solvents but distinct from that of fucose.

Its electrophoretic mobility in borate buffer pH 8.6[15] also agreed with that of rhamnose (Fig. 7).

These results thus provide sufficient evidence that the reducing residue of the isolated nucleotide is rhamnose.

The molar ratio of the reducing value to thymidine was 1 when the sugar was calculated as rhamnose, lending additional support to the above conclusion. The reducing value of rhamnose was about half that of glucose on a molar base.

Evidence for the attachment of phosphate at the 5′-position of thymidine: Upon hydrolysis of the nucleotide preparation in 1 *N* HCl at 100° for 10 min, a component with the composition and chromatographic mobility of TMP was liberated quantitatively. Treatment with bull semen 5′-nucleotidase converted this component to thymidine with liberation of phosphate (Fig. 8; Table IV). It is evident therefore that one of the phosphates in the compound is attached at the 5′ position of thymidine.

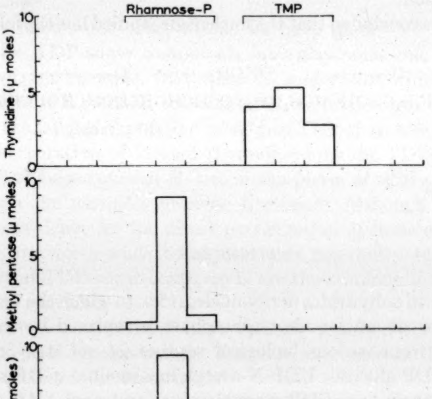
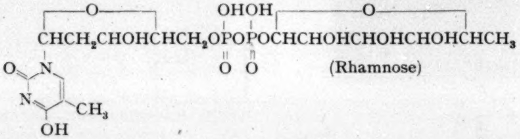


Fig. 9. Ion-exchange chromatography of phosphodiesterase digest of TDP-rhamnose. 2.25 μmoles TDP-rhamnose were incubated with 8 μmoles $MgCl_2$ and 0.04 unit[23] snake venom phosphodiesterase in a total volume of 4 ml at 37° for 30 min. During incubation the pH of the reaction mixture was maintained between 8.0–9.0 by occasional addition of NaOH. The reaction was stopped by adding TCA. After deproteinization and removal of TCA with ether, an aliquot of the sample was applied onto a column of Dowex-1-formate (X 10, 200–400 mesh), 0.6 × 17 cm. Gradient elution was carried out by introducing successively 50 ml of 1 *N* formic acid and 190 ml of 4 *N* formic acid into the mixing flask initially filled with 100 ml of water. 4.4 ml samples were collected at intervals of about 20 min. Analyses were made on each fraction after removal of formic acid by lyophilization. The amount of thymidine was calculated from extinction at 260 mμ.

Evidence for the pyrophosphate linkage between TMP and rhamnose-1-phosphate: The nucleotide was digested with purified snake venom phosphodiesterase which also possessed a strong nucleotide pyrophosphatase activity (tested by using DPN as substrate). Two phosphate-containing products were separated on Dowex-1 column (Fig. 9). One component possessed equimolar amounts of thymidine and phosphate and was eluted at the position corresponding to TMP, while the other component eluted just before the former, contained equimolar amounts of methylpentose and phosphate. The latter substance showed reducing activity only after liberation of phosphate by acid hydrolysis, indicating that the phosphate is substituted on the 1-position of the methylpentose. Thus it appeared that equimolar amounts of TMP and rhamnose-1-phosphate were released by the enzyme, probably by nucleotide pyrophosphatase activity. This fact provides evidence that in the compound, TMP is linked to rhamnose-1-phosphate through a pyrophosphate bond.

This conclusion is further supported by the fact that the hydrolysis with 0.01 *N* HCl at 100° for 10 min resulted in the liberation of TDP (with a smaller amount of TMP) as well as the formation of reducing group. TDP was characterized by analysis (thymidine/phosphate/reducing value = 1.00/2.00/0.00) and by conversion on hydrolysis with 1 *N* HCl at 100° to TMP, which was in turn identified as indicated in the previous section.

Conclusion: It is concluded that the nucleotide studied has the following structure:



DISCUSSION

A great number of acid anhydrides of ribonucleotides, in which the terminal phosphate or pyrophosphate of ribonucleoside triphosphate is replaced by some other group, have been isolated from various biological sources (*cf.* ref. 24). Some of these are UDP-sugars (*e.g.* UDP-glucose; UDP-N-acetylglucosamine), GDP-sugars (*e.g.* GDP-mannose), CDP-alcohols (*e.g.* CDP-ethanolamine) and acyl-AMP (*e.g.* amino acyl-AMP). Extensive studies have revealed that the major function of this type of compounds is in the activation of various groups for synthetic reactions.

Two acid anhydrides of deoxynucleotide were previously isolated: deoxy-CDP-choline from sea urchin eggs[25] and rat hepatoma[26], and deoxy-CDP-ethanolamine from calf thymus[27] and rat hepatoma[26]. An enzymic study[28] has shown that these compounds could be formed and utilized for the synthesis of lecithine or of phosphatidylethanolamine by the action of the same enzymes that catalyze the metabolism of the corresponding ribonucleotides. No result has been reported so far to assign a special metabolic role to these deoxynucleotide anhydrides.

The results presented in this paper leave little doubt that the nucleotide isolated from *L. acidophilus* is TDP-rhamnose. This is the first example of the isolation and identification of an acid anhydride of thymidine nucleotide.

After the publication of the preliminary report of the present work[4], STROMINGER AND SCOTT[29] have independently reported the isolation of three unidentified TDP-sugar compounds (TDP-X, TDP-Y and TDP-Z) from a diaminopimelic acid-requiring mutant of *Escherichia coli*. Two TDP-sugar compounds (TDP-X_1 and TDP-X_2) have also been isolated in our laboratory from *E. coli* strain B (Hershey), one of them (TDP-X_1) being identified as TDP-rhamnose[30]. The properties of all four unidentified sugars (STROMINGER'S X, Y and Z and our X_2) suggested that they might also be deoxysugars.

The metabolic functions of TDP-rhamnose and other TDP-sugar compounds call for special attention because of their possible relation to the function and metabolism of DNA.

By analogy with the metabolism of acid anhydrides of ribonucleotides such as UDP- and GDP-sugars, TDP-sugar compounds may well be considered to function as means of activation of sugars for synthesis of bacterial polysaccharides. In this connection it should be emphasized that rhamnose and other deoxysugars are known as constituents of antigenic polysaccharides of bacteria; *e.g.* rhamnose in capsular polysaccharides of type II *pneumococcus*[31], rhamnose and colitose in O antigen of *E. coli*[32, 33], and paratose, abequose and tyvelose in specific polysaccharides of group A, B and C *Salmonellae*[34].

On the other hand some observations we have made[1, 2, 35] suggest that TDP-rhamnose and other TDP-sugar compounds may play some rôle in DNA synthesis. It has been shown, for example, that when *L. acidophilus* which has been labeled with ^{3}H by exposure to a medium containing ^{3}H-thymidine was incubated in a non-labeled medium, the labeled thymidine moiety of TDP-rhamnose was transferred to DNA[2]. Active incorporation of labeled thymidine into the TDP-sugar fraction was also observed in T2-phage infected *E. coli* in the phase of active synthesis of phage DNA as well as in the normally growing bacteria[35]. Although these observations by no means give evidence for the direct participation of these compounds in DNA synthesis, it appears worth-while to explore this possibility further. It might be also conceivable that TDP-sugar compounds are the common intermediate of DNA and polysaccharide synthesis.

SMITH, GALLOWAY AND MILLS[36] have recently reported the isolation of UDP-rhamnose from type II *pneumococcus*. The relation between TDP-rhamnose and UDP-rhamnose remains to be investigated.

ACKNOWLEDGEMENTS

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REFERENCES

[1] R. Okazaki and T. Okazaki, *Biochim. Biophys. Acta*, 28 (1958) 470.
[2] R. Okazaki, T. Okazaki and Y. Kuriki, *Biochim. Biophys. Acta*, 33 (1959) 289.
[3] T. Okazaki and R. Okazaki, *Biochim. Biophys. Acta*, 35 (1959) 434.
[4] R. Okazaki, *Biochem. Biophys. Research Comm.*, 1 (1959) 34.
[5] R. Okazaki, T. Okazaki and Y. Kuriki, *Proc. 8th Symp. Nucleic Acids*, 1959, p. 21.
[6] R. B. Hurbept, H. Schmitz, A. F. Brumm and V. R. Potter, *J. Biol. Chem.*, 209 (1954) 23.
[7] H. A. Krebs and H. Hems, *Biochim. Biophys. Acta*, 12 (1953) 12.
[8] S. M. Partridge, *Nature*, 158 (1947) 270; *Biochem. J.*, 42 (1948) 238.
[9] E. W. Putman, in *Methods in Enzymology*, Vol. 3, Academic Press, Inc., New York, 1957, p. 62.
[10] L. M. White and G. E. Secor, *Arch. Biochem. Biophys.*, 43 (1953) 60.
[11] L. A. Jermyn and F. A. Isherwood, *Biochem. J.*, 44 (1949) 402.
[12] I. H. Pazur, *J. Am. Chem. Soc.*, 72 (1950) 5150.
[13] S. M. Partridge, *Nature*, 164 (1949) 443.
[14] R. Markham and J. D. Smith, *Biochem. J.*, 52 (1952) 552.
[15] R. Consden and W. W. Stanier, *Nature*, 169 (1952) 783.
[16] J. Baddiley, J. G. Buchanan, B. Carss, A. P. Mathias and A. R. Sanderson, *Biochem. J.*, 64 (1956) 599.
[17] J. B. Martin and D. M. Doty, *Anal. Chem.*, 21 (1949) 965.
[18] M. Levy and A. H. Palmer, *J. Biol. Chem.*, 136 (1940) 57.
[19] J. T. Park and M. J. Johnson, *J. Biol. Chem.*, 181 (1949) 149.
[20] Z. Dische and L. B. Shettles, *J. Biol. Chem.*, 192 (1951) 279.
[21] G. Ashwell, in *Methods in Enzymology*, Vol. 3, Academic Press, Inc., New York, 1957, p. 73.
[22] L. A. Heppel and R. L. Hilmoe, in *Methods in Enzymology*, Vol. 2, Academic Press, Inc., New York, 1957, p. 546.
[23] M. Privat De Garilhe and M. Laskowski, *Biochim. Biophys. Acta*, 18 (1955) 370.
[24] J. L. Strominger, *Physiol. Revs.*, 40 (1960) 55.
[25] Y. Sugino, *J. Am. Chem. Soc.*, 79 (1957) 5074; *Biochim. Biophys. Acta*, 40 (1960) 425.
[26] W. C. Schneider and Rotherman, *J. Biol. Chem.*, 233 (1958) 948.
[27] R. L. Potter and V. Buettner-Janusch, *J. Biol. Chem.*, 233 (1958) 462.
[28] E. P. Kenedy, L. F. Borkenhagen and S. W. Smith, *J. Biol. Chem.*, 234 (1959) 1998.
[29] J. L. Strominger and S. S. Scott, *Biochim. Biophys. Acta*, 35 (1959) 552.
[30] R. Okazaki, T. Okazaki and Y. Kuriki, *Biochim. Biophys. Acta*, 38 (1960) 384.
[31] P. W. Kent, *Chemistry & Industry*, 1952 (1952) 1176.
[32] O. Westphal and O. Lüderitz, *Angew. Chem.*, 66 (1954) 407.
[33] O. Lüderitz, A. M. Staub, S. Stirm and O. Westphal, *Biochem. Z.*, 330 (1958) 193.
[34] D. A. L. Davies, A. M. Staub, I. Fromme, O. Lüderitz and O. Westphal, *Nature*, 181 (1958) 822.
[35] T. Okazaki, Y. Kuriki, R. Okazaki and M. Sekiguchi, unpublished observation.
[36] E. E. B. Smith, B. Galloway and G. T. Mills, *Biochim. Biophys. Acta*, 33 (1959) 276.

AMINO ACID STARVATION IN AN *ESCHERICHIA COLI* AUXOTROPH

III. INCORPORATION OF ^{32}P INTO RIBONUCLEIC ACID AND OTHER CELL COMPONENTS

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SUMMARY

During amino acid starvation in leucine and proline auxotrophs of *E. coli*, although net protein and RNA synthesis have ceased, there is continuing incorporation of ^{32}P into RNA nucleotides. The turnover rate approximates 10 % per hour initially and is nearly equal in the debris fraction, the ribosomes, and the soluble supernatant. Both early and late in starvation and in each centrifugal fraction the specific radioactivities of the four RNA nucleotides are approximately equal. These findings are discussed with reference to the interrelation of RNA and protein synthesis and the cessation of both during amino acid starvation.

INTRODUCTION

It was shown in the preceding papers[1,2] that transfer of an exponentially growing leucine auxotroph of *E. coli* into a medium deficient in the required amino acid, results in immediate cessation of protein and RNA synthesis, while DNA synthesis continues to about 30 % net increase, and half the cells in the culture divide. The cells then remain viable for at least 90 min of starvation, and the machinery for protein and nucleic acid synthesis persists intact, for if leucine is again furnished, these macromolecular syntheses resume instantly at the former rate. The cells begin to divide again only after a lag of approximately half a generation time. During starvation the cells accumulate certain RNA precursors and related substances, notably ATP, UTP, UDPX compounds, CTP, DPN, uracil, and hypoxanthine.

It is by no means evident why normal RNA synthesis should be so completely dependent upon concurrent protein synthesis, although some speculative hypotheses have been advanced[3]. Since, as we have shown, the pathways leading to RNA precursors are plainly functional during amino acid starvation, and since the precursors themselves are abundant, the cause of the block must be sought further on,

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Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; ATP, adenosine triphosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate; DPN, oxidized diphosphopyridine nucleotide; GMP, guanosine monophosphate; UMP, uridine monophosphate; CMP, cytidine monophosphate; AMP, adenosine monophosphate; BD, bacterial density; PCA, perchloric acid.

in the mechanisms concerned with the sequential arrangement and polymerization of the nucleotides. The studies reported here were therefore undertaken to see whether or not nucleotide precursors could be incorporated into RNA during amino acid starvation, despite the complete blockade of net RNA synthesis. Some of the findings have been reported in a preliminary communication[4].

METHODS

Growth and starvation

Leucine and proline auxotrophs of *E. coli* K12 were used as described previously[1]. Cells were grown at 37° in 1.5–3 l aerated mineral-glucose medium containing sufficient leucine (or proline), harvested at B.D. 0.12 g dry wt./l, washed twice with 250-ml portions of 0.15 % NaCl, and resuspended to the same B.D. in medium lacking the required amino acid. Samples were then withdrawn periodically during starvation. When originally harvested from the complete medium the cells were growing exponentially and had only attained one-quarter of the final B.D. that the medium will support. If the washed cells are resuspended in a complete medium instead of a deficient one, they resume growth immediately at the former rate. That the cells remain competent through at least 90 min starvation was shown in refeeding experiments[1].

3 mC of sodium radiophosphate (Abbott Laboratories, Oak Ridge) was used in each experiment. The contaminant non-orthophosphate ^{32}P which we describe elsewhere[5] did not significantly affect the data reported here because the nucleotides were adequately separated from non-nucleotide P, and because (in the later experiments) charcoal columns were used, as described below. In one type of experiment cells uniformly labelled by growth in $^{32}PO_4$ were resuspended in non-radioactive amino acid-deficient medium. A balance of ^{32}P exchanges among the various cellular fractions during starvation could thus be obtained, as well as a direct measure of ^{32}P excretion. In another type of experiment ordinary cells were starved in radioactive medium so that ^{32}P incorporation could be followed. For radioactivity determinations, appropriately diluted samples were dried on stainless steel planchets and counted with a thin-window G-M tube; counting rates in excess of 5 times background were usually achieved. Since the medium contained 63.5 mmoles P/l while total cell P was only 150 μmole/l, the uptake of ^{32}P by the cells could not appreciably lower the medium specific radioactivity. The specific activity of each cell component was compared with that of the whole culture (cells + medium). Incorporation was expressed as specific activity of sample × 100/specific activity of culture. Since there was little or no net synthesis of RNA during starvation, the incorporation data signify, in effect, % turnover.

Centrifugal fractionation

The harvested cells were washed in cold 0.15 % NaCl and resuspended in 50 ml of 0.003 *M* $MgSO_4$. The B.D. of this suspension was measured turbidimetrically and an aliquot removed for chemical analyses (whole cell fraction). The remainder was treated in the cold for 2 min in a Raytheon 10 kc oscillator at maximum power, then centrifuged at 3,000 × *g* for 10 min to remove unruptured cells. The sediment was resuspended and assayed by turbidimetry or determination of total PCA insoluble P. These measures agreed well in estimating the fraction of cells that escaped rupture, from which could be calculated the dry wt. in the sonic lysate. The 3,000 × *g* supernatant was spun at 26,000 × *g* for 1 h, yielding a debris fraction; 105,000 × *g* for 1 h gave a sediment largely composed of 70 S particles (ribosome fraction) and a soluble fraction supernatant. (For the analytical ultracentrifuge runs we are indebted to Dr. F.-C. CHAO.)

Chemical fractionation

Aliquots of each centrifugal fraction (or of whole cells in some of the experiments) were made to 6 % with cold PCA, centrifuged after standing 1 h in ice, and the precipitates washed once with PCA. Phospholipids were removed with water–ethanol, ethanol–ether, and ether, and the residues were made up in 5 ml of 1 *N* KOH and incubated 16 h at 37°. A blank tube containing KOH was also incubated, carried through all subsequent steps, and used to correct the experimental data. The tubes were chilled, exactly neutralized with PCA, then made to 6 % with additional PCA and centrifuged cold to remove $KClO_4$, DNA, and protein. The supernatants, containing RNA nucleotides and non-nucleotide ("concomitant") P, were filtered through PCA-washed paper and each combined with a single cold PCA wash. DNA nucleotides (and "concomitant" P) were extracted from the residues with three 1.5-ml portions of 6 % PCA at 90°; the extracts were chilled, neutralized with KOH, centrifuged, filtered, and made up to 5 ml with water. The protein residues were dissolved in 1 *N* KOH. The cold acid-soluble, phospholipid, RNA, DNA, and protein fractions were assayed for absorbance at 260 mμ, total P (see ref. 6) and ^{32}P; however, as indicated in the data, not all chemical fractions were analyzed in every experiment.

We show elsewhere[5] that non-nucleotide P may seriously distort the RNA incorporation data, especially in the debris fraction. In most of the experiments, therefore, the RNA nucleotides were freed of such material on charcoal. Columns (1 × 11 cm) were prepared with a bottom layer of Whatman cellulose powder, then a 1:3 mixture of Norit A and cellulose powder, and finally a top layer of cellulose powder. The columns were operated under 0.2 atm. positive pressure. The cellulose powder prevented dispersion or leakage of charcoal and permitted adequate flow rates to the end of the run, but the columns could not be used again. The columns were washed well with 2 *M* NH_4OH in 50 % ethanol, then water, then 6 % PCA, and the RNA nucleotide solutions in PCA were poured on. The P and ^{32}P contents of the combined run-through and water washes were determined: these solutions absorbed negligibly at 260 mμ. The adsorbed mixture of nucleotides could be recovered quantitatively by eluting with sufficiently large volumes of ammonia–ethanol; in these experiments, however, the total eluate volumes were limited to 40 ml, giving recoveries of about 80 %. The eluates were taken to dryness in a flash-evaporator in the cold, then redissolved in water as required for the various determinations, or for separation of nucleotides.

Isolation of RNA nucleotides

The nucleotide mixture was applied to a 30 × 30 cm square of Whatman 3MM paper and run as an ascending chromatogram in a modified formulation of MARKHAM's[7] solvent 5 (solvent 5f: 700 ml isopropanol, 63 ml conc. formic acid, water to 1 l). In some of the early experiments the charcoal column was omitted and the alkaline digest was neutralized, filtered, dried, redissolved, and directly chromatographed. Electrophoresis was then carried out in the second dimension in 0.1 N formic acid (pH 2.6, 16 V/cm) for 2.5 h. Each of the 3 rather large u.v. quenching spots was cut out, eluted with 0.1 M NH_4OH onto a strip of 3MM paper, and subjected to ascending chromatography in an ammonium sulfate–isopropanol solvent (300 g $(NH_4)_2SO_4$ in 500 ml water, 10 ml isopropanol, 6 g Tris, pH 7). This final chromatogram confirmed the homogeneity of 2 bands (GMP, UMP) and resolved the 3rd into well-separated spots of CMP and AMP. In later experiments it was found that satisfactory separations could be obtained in a single 2-dimensional run consisting of electrophoresis in formic acid as described above, followed by ascending chromatography in saturated ammonium carbonate. The electrophoretic separation gives (in order, from the anode) well separated spots of UMP, GMP and an AMP–CMP mixture. The chromatogram gives (in order of ascending R_F) AMP (2 spots), GMP, and a CMP–UMP mixture, all well separated. The 2 solvent systems not only complement each other from the standpoint of nucleotide separation, but offer the additional advantage of complete volatility. The spots were finally eluted and *u.v.* spectra recorded against appropriate paper blanks at acid and alkaline pH in the Beckman DK recording spectrophotometer. The spectra corresponded in all significant details with those of authentic 2′,3′-nucleoside monophosphates. The solutions were also assayed for P and ^{32}P. Further proof of purity was afforded by the ratio $O.D._{260}/P$ for each nucleotide.

RESULTS

Phosphate exchanges during amino acid starvation

Cells of the leucine auxotroph were labelled uniformly by growth in $^{32}PO_4$, then resuspended in non-radioactive medium lacking leucine, and harvested after 90 min. A typical experiment is shown in Table I. The characteristic chemical changes described previously are evident here. B.D. increased 17 % but protein N and RNA negligibly or not at all. (In other experiments the same changes often occurred without any increase of turbidity.) Acid-soluble and phospholipid P increased markedly, acid-soluble u.v.-absorbing substances somewhat less than P. Very considerable quantities of u.v.-absorbing substances were excreted from the cells. (Compare 154 A.U.*/l excreted during starvation with 144 A.U./l in the medium in which exponential growth had occurred.) There was net synthesis of DNA to about 30 % increase as measured by P or u.v. absorption. Protein residue P increased somewhat. The viable count increased during starvation to the same extent in ^{32}P as in ordinary medium.

The fate of P present in the cells at the onset of starvation can be followed in the 3rd column of the table. Except for DNA, all fractions lost ^{32}P and the aggregate loss was accounted for by ^{32}P appearing in the medium. The greatest amount of ^{32}P was lost from RNA, but the net loss of RNA was negligible (in most experiments zero). Thus about 12 % of the RNA finally present was derived from newly-formed precursors during the course of starvation. In contrast, DNA present before starvation was conserved. All estimates of the incorporation of P during starvation are neces-

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* 1 A.U. = amount of material in 1 ml of solution with $O.D._{260}$ = 1.0.

TABLE I

PHOSPHATE EXCHANGES DURING LEUCINE STARVATION

Cells were grown for 3 h with adequate leucine in medium containing $^{32}PO_4$, then harvested and washed twice. Half the cells were used for pre-starvation assays, the other half were resuspended for 90 min in non-radioactive medium without leucine. In the experiment shown here, B.D. increased during starvation from 0.124 to 0.146, protein N from 12.3 to 12.9 mg/l. All data given are per l culture. Data of columns A and B are based on counts and specific activity (53.5 counts/sec/μmole P) in the uniformly labelled cells; data of column C are from chemical determinations on the post-starvation cells. Upper part of table: P, as μmoles; lower part of table: absorbance units (1 A.U. = amount of material in 1 ml of solution with $O.D._{260} = 1.0$).

	(A) *P before starvation*	*(B)* *Old P still present after starvation*	*(A)–(B)* *Loss of old P*	*(C)* *Total P after starvation*	*(C)–(B)* *Gain of new P*	*(C)–(A)* *Net gain of P*
Whole cells	116	103	13	136	33	20
Acid-soluble	8.40	4.37	4.03	15.8	11.43	7.40
Phospholipid	11.7	9.33	2.37	17.5	8.17	5.8
RNA	80.5	69.1	11.4	78.5	9.4	—2.0
DNA	19.6	19.3	0.3	25.6	6.3	6.0
Protein residue	1.84	1.38	0.46	2.04	0.66	0.20
Sum of fractions	122	104	19	139	36	17
Old P in culture filtrate after starvation			22			

	A.U. before starvation	*A.U. after starvation*	*Net gain of A.U.*
Acid-soluble	38.4	59.6	21.2
RNA	786	831	45
DNA	141	184	43
Culture filtrate	0	154	154

sarily minimum since incorporation of ^{32}P from the pre-starvation pool would not have been detected.

Phosphate incorporation into RNA during starvation

In order to ascertain whether the observed turnover of P in the RNA fraction during starvation actually represented a turnover of RNA nucleotides, cells were grown in nonradioactive medium and starved in the presence of $^{32}PO_4$. RNA nucleotides were then isolated as described and their specific activity compared with that of P in the culture medium. Typical experiments with leucine and proline auxotrophs are shown in Table II. Absence of concomitant P in the isolated mononucleotides is indicated by the good agreement of data in the first 2 columns. The final column shows that the extent of incorporation of all 4 nucleotides was the same, within experimental error. On the other hand, RNA turnover was consistently less in proline than in leucine starvation. We do not know whether this has anything specific to do with proline lack as against leucine lack, or whether (as seems more likely) it arises from a non-specific difference between the 2 auxotrophic strains.

Cultures starved in the presence of $^{32}PO_4$ were sampled periodically in order to obtain information on the rate of RNA turnover. Typical of such experiments with

TABLE II

PHOSPHATE INCORPORATION INTO RNA DURING LEUCINE AND PROLINE STARVATION

Cells were grown for 3 h with adequate amino acid; in the first experiment a leucine auxotroph, in the 2nd a proline auxotroph was used. The cells were washed twice and resuspended for 90 min in amino acid-deficient medium containing $^{32}PO_4$. Culture was sampled initially and finally for B.D., protein N, ribose, and viable count. RNA fraction and individual RNA nucleotides were obtained as described under METHODS. Initial and final data in the leucine experiment were: B.D. 0.157, 0.157; protein N 16.0, 16.2 mg/l; ribose 74, 78 μmoles/l; viable 261, 470·10^9/l. In the proline experiment the corresponding data were: B.D. 0.131, 0.142; protein N 13.5, 14.4; ribose 65, 68; viable 273, 414. Published data on ε_{260}(P) are from BEAVEN *et al.*[8]. Experimental data are from O.D.$_{260}$ and P determinations. For ^{32}P counting 1-ml samples were plated. Nucleotide solutions did not require dilution; RNA samples were diluted 1:10, whole culture samples 1:1331. Data are corrected for background (0.34 counts/sec), decay, and dilution. In the last column incorporation is expressed as 100 × specific activity of sample/specific activity of whole culture; in the absence of net change this represents turnover of RNA as % of that present.

Sample	*ε_{260}(P) published*	*ε_{260}(P) found*	*Counts/sec/ml*	*μmole P/ml*	*Specific activity Counts/sec/μmole P*	*Incorporation %*
Leucine starvation						
Whole culture	—	—	4672	53.1	88.0	—
RNA fraction	—	—	117	8.86	13.2	15.0
CMP 1.	6.8	6.4	5.23	0.383	13.7	15.6
2.		6.4	5.74	0.430	13.3	15.1
AMP 1.	14.2	14.6	4.82	0.377	12.8	14.6
2.		14.1	5.08	0.411	12.4	14.1
GMP 1.	11.8	11.9	4.81	0.360	13.4	15.2
2.		11.6	4.58	0.365	12.6	14.3
UMP 1.	10.0	9.9	4.84	0.359	13.5	15.3
2.		10.0	3.92	0.303	13.0	14.8
Proline starvation						
Whole culture	—	—	5192	54.0	96.1	—
RNA fraction	—	—	87.3	8.41	10.4	10.8
CMP	6.8	6.6	3.87	0.363	10.7	11.1
AMP	14.2	14.2	4.16	0.377	11.0	11.4
GMP	11.8	12.1	3.45	0.306	11.3	11.7
UMP	10.0	9.9	3.93	0.358	11.0	11.4

leucine and proline auxotrophs is that shown in Fig. 1. Turnover began at the onset of starvation and continued at a decreasing rate, for as long as 3 h. The initial rate of turnover in leucine starvation approximated 10 % per hour. The decline in the slope of the curve with time, while so little of the RNA has yet turned over, suggests the possibility that the asymptote is not 100 % but some smaller fraction.

RNA turnover in various centrifugal fractions

The RNA of different particle sizes is known to differ functionally. Ribosomes are believed to be sites of protein synthesis[9]. The soluble fraction contains "transfer RNA" which accepts activated amino acids and presumably participates in their sequential arrangement on ribosomal templates[10]. Debris RNA is less well understood but may represent ribosomes bound to membrane fragments[9]. In our experience[5] the RNA of *E. coli* is distributed between debris, ribosome and soluble fractions in the approximate ratio 1:4:1. The ribosomal RNA is unchanged during amino acid starvation (Table III), and there was also no change in the amounts of 30 S and 50 S material (from dissociated 70 S particles[11]) in analytical ultracentrifuge runs at 0.001 M Mg^{++} and below. Which of the fractions are involved in RNA turnover and at what relative rates? Some typical experiments are summarized in Table IV. Both early and late in starvation RNA turnover occurred in all the centrifugal fractions.

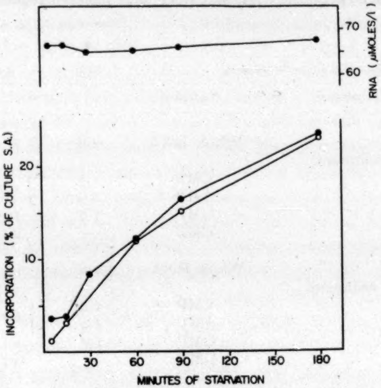

Fig. 1. Kinetics of ^{32}P incorporation into RNA during amino acid starvation (leucine auxotroph). Cells grown 3 h in adequate leucine, washed and resuspended in leucine-deficient medium containing $^{32}PO_4$. Samples taken as shown and worked up for RNA and combined CMP + AMP derived from RNA, as described under METHODS. ●—●, RNA, based on O.D.$_{260}$ and $\varepsilon_{260} = 12.0$; upper curve, right ordinate, total cell RNA, µmole/l; lower curve, left ordinate, incorporation, % of RNA turned over. ○—○, CMP + AMP, based on P. All counts except some at 5 and 15 min were greater than 5 times background.

TABLE III

STABILITY OF RIBOSOME RNA DURING LEUCINE STARVATION

A culture was sampled during exponential growth at the time of harvesting, then periodically after resuspension of the washed cells in leucine-deficient medium. The procedures described under METHODS were followed for sonic rupture, centrifugal fractionation and chemical isolation of RNA in each sample. Ribose (by the orcinol reaction) and O.D.$_{260}$ were then determined. Data are µmoles ribose and absorbance units per g original cells.

	Ribosome fraction	
	Ribose	*A.U.*
Exponential growth	155	3180
15 min starved	157	3080
90 min starved	155	2950

In all experiments and at all times during starvation, although the differences were small, turnover was greatest in the soluble and least in the ribosome fraction. The minor differences between nucleotides within a single fraction were not consistent and are not thought to be real.

TABLE IV

PHOSPHATE INCORPORATION INTO RNA OF VARIOUS CENTRIFUGAL FRACTIONS DURING LEUCINE STARVATION

Procedures as described under METHODS. In this experiment 80% of the cells were lysed by 2 min sonic oscillation. Data are % turnover, calculated as 100 × specific activity of sample/specific activity of whole culture; since there was no net change in RNA this is equivalent to % turnover The data are from 3 different experiments. In Expt. a the UMP was not isolated; in Expt. b the CMP and AMP were isolated together.

Expt.		*a*	*b*	*c*
Duration of starvation (min)		*10*	*10*	*90*
Centrifugal fraction	*Nucleotide*	*% turnover*		
Debris (26,000 × *g* sediment)	Whole RNA	2.0	—	10.5
	CMP	1.7	1.7	
	AMP	1.9		
	GMP	1.7	1.9	
	UMP	—	2.0	
Ribosomes (105,000 × *g* sediment)	Whole RNA	1.6	—	10.0
	CMP	1.2	1.1	
	AMP	1.4		
	GMP	1.2	1.1	
	UMP	—	0.7	
Soluble (105,000 × *g* supernatant)	Whole RNA	2.6	—	13.7
	CMP	2.1	1.5	
	AMP	2.2		
	GMP	2.0	1.4	
	UMP	—	1.7	

DISCUSSION

The experiments reported here show that during amino acid starvation in *E. coli* auxotrophs, after net synthesis of RNA and protein have ceased, there is continued incorporation of ^{32}P from the medium into the nucleotides of RNA. About 10% of the RNA is thus renewed during the first hour. The conservation of DNA phosphate speaks against a simultaneous lysis and re-synthesis of whole cells, and MANDELSTAM[12] has shown in another way that lysis is negligible under these conditions. Nor is there any general degradation of RNA or of ribosomes. In this respect amino acid starvation in an otherwise complete medium differs from incubation in buffer alone[13]. Finally, the magnitude of the turnover makes it evident that we are not dealing primarily with an exchange of terminal nucleotides. Our data on the rate of RNA turnover are not unlike those of GROS AND GROS[14], although they considered the observed incorporation of ^{32}P and [^{14}C]adenine to be negligible.

Thus it appears that phosphodiester bonds in RNA may be labilized to permit nucleotide exchange, or that entire RNA molecules are depolymerized and resynthesized. Either interpretation bespeaks the presence and continued functioning of templates for RNA synthesis during amino-acid starvation. The near-equality of specific radioactivities in the debris, ribosome, and soluble fractions suggests parallel and independent turnover of the different types of RNA. About 4 nucleotides exchange in the ribosomes for each one in the soluble or debris fraction. Since this is precisely the synthetic ratio during balanced growth, it may be that the turnover process represents the continued operation during starvation of those mechanisms that would ordinarily result in net RNA synthesis.

We showed previously[2] that there is an ample supply of nucleoside di- and triphosphates, and it now appears that their arrangement and polymerization into RNA can and do occur during amino-acid starvation; yet the total amount of RNA does not increase. The cessation of RNA synthesis on withholding a single required amino acid (and of protein synthesis when a single pyrimidine is absent) have been attributed to the need for amino-acid-nucleotide compounds as common intermediates in both synthetic pathways[14]. It is evident, however, from MANDELSTAM's[12] experiments, that during starvation the deficient amino acid should be available within the cell as a result of protein turnover. The hypothetical common intermediates should then also be present, and one should see a continued net synthesis of RNA and ribonucleoprotein at the expense of soluble protein. Likewise, when chloramphenicol blocks protein synthesis in an amino-acid auxotroph, RNA synthesis will not proceed unless the deficient amino-acid is supplied. That which is derived from protein turnover does not suffice, but traces furnished in the medium are quite adequate[15]. These findings suggest that protein turnover may not actually yield amino acids in the form required for RNA synthesis. Alternatively, the turnover of RNA and protein may be tightly coupled so that all protein degradation within the cell is accompanied by an equivalent depolymerization of RNA, making net synthesis impossible.

In MANDELSTAM's[12] experiments amino acid residues of protein were replaced at about 5 %/h, representing 2 % of cell dry wt. In our experiments RNA nucleotides were replaced at 10 %/h, which also amounts to 2 % of cell dry wt. Assuming an average nucleotide to be 3 times heavier than an average amino acid, it follows that 3 amino acids are replaced in protein for each nucleotide replaced in RNA. It has been proposed that much of a cell's RNA may be the inert end-product of "co-synthesis", oligonucleotide "coding units" being polymerized after carrying amino acids to the sites of protein synthesis. For the continued cycling of such a process during starvation we should expect a turnover ratio (nucleotides: amino acids) of at least 3:1, a trinucleotide being the smallest possible "coding unit". The observed ratio (1:3) departs very widely from this value. On the other hand, a coupled turnover during starvation might be expected to reflect the normal synthetic ratio of nucleotides in RNA to amino acids in protein during balanced growth (about 1:8 in this strain), but here the observed ratio falls conspicuously short.

The equal specific radioactivity of all four nucleotides in RNA, even early in starvation when only 1–2 % have exchanged, requires explanation. We have shown[2] that the nucleotide pools of adenine and uracil are much larger than those of guanine and cytosine. It might be expected, therefore, that newly-formed RNA precursors would be diluted unequally prior to their incorporation. The equal specific activities observed in our experiments may therefore indicate (as also proposed recently for mammalian cells[16]) that each of the four pools turns over at a rate proportional to its own size; *i.e.*, the pool size is determined by the cell's total need for a particular nucleotide. In support of this generalization is the fact that the larger pools are just those which are called upon to supply nucleotides for the more abundant coenzymes as well as for the nucleic acids.

MANDELSTAM[17] has pointed out the probable survival value of protein turnover in providing amino acids for the initial expanded synthesis of repressed enzymes in sudden shifts from rich to deficient media. Since enzyme biosynthesis requires a supply of nucleotides as well as amino acids, RNA turnover could serve an analogous purpose in sudden "shifts down" to purine and pyrimidine deficiency. Related problems have been investigated extensively by MAALOE[18].

ACKNOWLEDGEMENT

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REFERENCES

[1] A. GOLDSTEIN, D. B. GOLDSTEIN, B. J. BROWN AND S.-C. CHOU, *Biochim. Biophys. Acta*, 36 (1959) 163.
[2] D. B. GOLDSTEIN, B. J. BROWN AND A. GOLDSTEIN, *Biochim. Biophys. Acta*, 43 (1960) 55.
[3] F. H. C. CRICK, *Symposia Soc. Exptl. Biol.*, 12 (1958) 138.
[4] A. GOLDSTEIN, D. B. GOLDSTEIN AND B. J. BROWN, *Federation Proc.*, 17 (1958) 372.
[5] A. GOLDSTEIN AND B. J. BROWN, *Biochim. Biophys. Acta*, (to be submitted).
[6] H. S. LORING, H. W. BORTNER, L. W. LEVY AND M. L. HAMMELL, *J. Biol. Chem.*, 196 (1952) 807.
[7] R. MARKHAM, *Brit. Med. Bull.*, 10 (1954) 214.
[8] G. H. BEAVEN, E. R. HOLIDAY AND E. A. JOHNSON, in E. CHARGAFF AND J. N. DAVISON, *The Nucleic Acids*, Vol. I, Academic Press, New York 1955, p. 493.
[9] K. MCQUILLEN, R. B. ROBERTS AND R. J. BRITTEN, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 1437.
[10] P. BERG AND E. J. OFENGAND, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 78.
[11] A. TISSIERES, J. D. WATSON, D. SCHLESSINGER AND B. R. HOLLINGWORTH, *J. Mol. Biol.*, 1 (1959) 221.
[12] J. MANDELSTAM, *Biochem. J.*, 69 (1958) 110.
[13] J. MENDELSOHN AND A. TISSIERES, *Biochim. Biophys. Acta*, 35 (1959) 248.
[14] F. GROS AND F. GROS, *Exptl. Cell Research*, 14 (1958) 104.
[15] A. B. PARDEE AND L. S. PRESTIDGE, *J. Bacteriol.*, 71 (1956) 677.
[16] L. L. BENNETT, JR., H. E. SKIPPER, L. SIMPSON, G. P. WHEELER AND W. S. WILCOX, *Cancer Research*, 20 (1960) 62.
[17] J. MANDELSTAM, *Nature*, 179 (1957) 1179.
[18] O. MAALOE, *10th Symposium, Society for General Microbiology*, Cambridge Univ. Press, Cambridge, 1960.

REDUCED PYRIDINE NUCLEOTIDES AS ACTIVATORS OF CERTAIN REACTIONS CATALYZED BY PEROXIDASE

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SUMMARY

Conditions are described in which the aerobic oxidation of epinephrine and ferrocytochrome *c* by peroxidase is dependent on the presence of DPNH or TPNH in the reaction mixture and is stimulated by thyroxine, certain thyroxine analogues and estradiol. In the case of epinephrine oxidation, DPNH and thyroxine abolished a lag period which is associated with the aerobic oxidation of epinephrine in the absence of these substances. The oxidation of epinephrine and ferrocytochrome *c* by peroxidase in the presence of Mn^{++}, DPNH, thyroxine and oxygen is inhibited by catalase and by certain thyroxine analogues. The latter inhibition is overcome by an increase in DPNH concentration.

Thyroxine is inactivated by the H_2O_2–peroxidase system and this inactivation is inhibited by the presence of a suitable hydrogen donor (*e.g.* DPNH, epinephrine) in the reaction mixture. A DPNH dependent inactivation of thyroxine by the Mn^{++}-peroxidase–oxygen system also is described.

It is suggested that the Mn^{++} dependent aerobic oxidation of reduced pyridine nucleotides by peroxidase facilitates certain secondary oxidations as a result of the generation of hydrogen peroxide in the course of the reaction.

INTRODUCTION

Thyroxine has been found to stimulate greatly the oxidation of a number of hydrogen donors by the H_2O_2–peroxidase system[1,2]. The suggestion has been made[1,2] that thyroxine facilitates peroxidatic reactions by acting as an electron carrier, being alternately oxidized by the H_2O_2–peroxidase system and reduced by the hydrogen donor. The phenolic hydroxyl group of thyroxine is essential for this effect. Certain hydrogen donors are oxidized by peroxidase in the absence of added H_2O_2, if Mn^{++} and oxygen are present in the reaction mixture (see ref. 3 for early bibliography). DPNH and TPNH are among the hydrogen donors which are oxidized by peroxidase in this fashion[3]. Thyroxine[2] and estradiol[2,4] as well as other phenolic compounds[3] stimulate the Mn^{++} dependent aerobic oxidation of DPNH and TPNH by peroxidase.

In the present study thyroxine and estradiol sensitive oxidations are described which require peroxidase, manganese and oxygen but which differ from the classical

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Abbreviations: TPNH, reduced triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

Mn^{++} dependent aerobic oxidations by peroxidase in that the addition of DPNH or TPNH also is required.

METHODS AND MATERIALS

Ferricytochrome *c* (Sigma Chemical Co.) was reduced with sodium borohydride and the latter was removed by dialysis[5]. The horseradish peroxidase (RZ 1.1) was obtained from Worthington Biochemical Corp. and the myeloperoxidase was prepared as previously described[1]. The myeloperoxidase solution employed had a ratio 430 mμ/390 mμ of 1.62 and an O.D. at 430 mμ of 0.090. All other materials were obtained as previously described[1,2]. The thyroxine analogues were kindly supplied by Drs. R. C. KROC AND A. W. RUDDY of the Warner-Lambert Research Institute. The spectrophotometric measurements were made at 25° with a Cary M 14 recording spectrophotometer. The blank was air unless otherwise indicated.

RESULTS

Epinephrine oxidation

The oxidation of epinephrine by H_2O_2 and peroxidase is stimulated by thyroxine[1]. Estradiol also stimulates this reaction and, as in the case of thyroxine, the stimulation is a function of the phenolic hydroxyl group. Fig. 1 demonstrates that for the immediate oxidation of epinephrine by peroxidase in the absence of added H_2O_2, DPNH was required in addition to Mn^{++} and thyroxine or estradiol. DPNH could be replaced by equimolar concentrations of TPNH. The concentration of DPNH required for the oxidation of epinephrine under the conditions employed in Fig. 1 was higher in the estradiol stimulated system than in the thyroxine stimulated system. At a DPNH

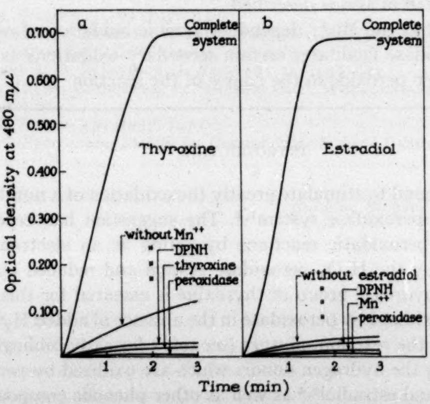

Fig. 1. Stimulation by thyroxine or estradiol of the oxidation of epinephrine by peroxidase in the presence of Mn^{++}, DPNH and oxygen. The complete system contained 120 μmoles of phosphate buffer pH 7.0, 0.5 μmole of epinephrine, 0.5 μmole of manganous chloride, 50 μg of horseradish peroxidase and water to a final volume of 3.0 ml. In addition *a* contained 0.3 μmole of DPNH and 0.05 μmole of thyroxine, whereas *b* contained 1.2 μmole of DPNH, 0.05 μmole of estradiol and ethanol to a final concentration of 1.7 %. The reaction was begun by the addition of peroxidase. The blank contained all components except peroxidase.

concentration of 0.3 μmole/3.0 ml of reaction mixture, epinephrine was rapidly and completely oxidized by the peroxidase system in the presence of thyroxine whereas only a slight oxidation was observed in the presence of estradiol. Approximately 1.2 μmoles of DPNH were required to produce a complete oxidation of epinephrine by the estradiol stimulated system.

The oxidation of epinephrine by peroxidase, Mn^{++}, DPNH and thyroxine was associated with an oxygen uptake and could be followed manometrically. Under the conditions employed, epinephrine was oxidized in the absence of DPNH and thyroxine, at a rate equal to that observed in the presence of DPNH and thyroxine following a lag period of approx. 30 min. The lag period was completely abolished on the addition of DPNH and thyroxine but not by either alone. In the absence of DPNH, 5 μmoles of oxygen were taken up during the oxidation of 2.0 μmoles of epinephrine following the lag period indicating that several oxygen consuming steps occur during the oxidation of epinephrine. If thyroxine (0.1 μmole) and DPNH (2.0 μmoles) also were added to the reaction mixture, the oxygen uptake was increased to 6.5 μmoles.

Catalase inhibited the oxidation of epinephrine by peroxidase in the presence of Mn^{++}, DPNH, thyroxine and oxygen. In a typical experiment the rate of oxidation of epinephrine was inhibited 90% by 800 μg of catalase, 17% by 800 μg of heated catalase (10 min at 100°) and 12% by 800 μg of crystalline bovine albumin.

Of a number of thyroxine analogues tested at a final concentration of $1.7 \cdot 10^{-5} M$, 3,5-diiodo-L-tyrosine, *n*-butyl 3,5-diiodo-4-hydroxybenzoate, DL-thyronine and 3,5-diiodo-L-thyronine had very little effect on epinephrine oxidation under the conditions employed in Fig. 2, whereas 3,3′,5-triiodo-L-thyronine and 3,3′,5′-triiodo-DL-thyronine were approximately half as active as thyroxine. An increase in the concentration of thyronine or diiodothyronine to 20 times that employed in Fig. 2 had little effect on the oxidation of epinephrine whereas a stimulation was produced

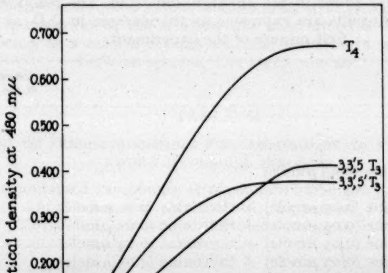

Fig. 2. Effect of thyroxine analogues. As in Fig. 1a except that 0.05 μmole of thyroxine (T_4), 3,3,5′-triiodo-L-thyronine (3,3,5′ T_3), 3,3′5′-triiodo-DL-thyronine (3,3′5′ T_3), 3,5-diiodothyronine (DITh), thyronine (Th), 3,5-diiodotyrosine (DIT) and *n*-butyl-3,5-diiodo-4-hydroxybenzoate (BDHB) were added as indicated.

by high concentrations of diiodotyrosine and *n*-butyl 3,5-diiodo-4-hydroxybenzoate. These latter analogues were less than 1 % as active as thyroxine.

Thyronine, at concentrations equimolar to that of thyroxine, largely prevented the stimulation of epinephrine oxidation by thyroxine under the conditions employed in Table I. An inhibition also was observed with 3,5-diiodothyronine and 3,3'5'-triiodothyronine although higher concentrations of these analogues were required. The antithyroxine effect of these analogues was abolished by an increase in the DPNH concentration.

Ferrocytochrome c *oxidation*

ALTSCHUL, ABRAMS AND HOGNESS[6] described the presence in yeast of a peroxidase with a very high affinity for ferrocytochrome *c*. This was confirmed by CHANCE[7], who also observed that the oxidation of ferrocytochrome *c* was a general property of peroxidase whether isolated from horseradish, milk, white blood cells or yeast. Fig. 3 demonstrates the stimulatory effect of thyroxine on the oxidation of ferrocytochrome *c* by horseradish peroxidase and H_2O_2. The product of oxidation was ferricytochrome *c* as indicated by its absorption spectrum and by its reduction to ferrocytochrome *c* on the addition of ascorbic acid (Fig. 3).

Added hydrogen peroxide could be replaced by the glucose–glucose oxidase system, horseradish peroxidase by myeloperoxidase and thyroxine by a number of thyroxine analogues or by estradiol. Thyronine was approximately 10 times and estradiol 4 times as active as thyroxine on a molar basis when horseradish peroxidase was employed, whereas thyroxine was approx. 1.5 times as active as thyronine, and thyroxine and estradiol were equally active when myeloperoxidase was employed.

TABLE I

ANTITHYROXINE EFFECT OF CERTAIN THYROXINE ANALOGUES

As in Fig. 1a except that thyroxine, thyronine, 3,5-diiodothyronine (DITh) and 3,3'5'-triiodo-DL-thyronine (3,3'5' T_3) were added in the amounts indicated. The reaction was begun by the addition of peroxidase. The results are expressed as the increase in O.D. at 480 mμ during the first minute of the experiment.

Additions	*Increase in O.D. at 480 mμ in 1 min*
None	0.010
DPNH	0.010
DPNH + thyroxine (0.05 μmole)	0.330
DPNH + thyroxine (0.05 μmole) + thyronine (0.01 μmole)	0.245
DPNH + thyroxine (0.05 μmole) + thyronine (0.02 μmole)	0.125
DPNH + thyroxine (0.05 μmole) + thyronine (0.03 μmole)	0.090
DPNH + thyroxine (0.05 μmole) + thyronine (0.05 μmole)	0.060
DPNH + thyroxine (0.05 μmole) + thyronine (0.1 μmole)	0.045
DPNH + thyroxine (0.05 μmole) + DITh (0.05 μmole)	0.420
DPNH + thyroxine (0.05 μmole) + DITh (0.2 μmole)	0.290
DPNH + thyroxine (0.05 μmole) + DITh (0.5 μmole)	0.145
DPNH + thyroxine (0.05 μmole) + DITh (1.5 μmoles)	0.95
DPNH + thyroxine (0.05 μmole) + 3,3'5'T_3 (0.05 μmole)	0.495
DPNH + thyroxine (0.05 μmole) + 3,3'5'T_3 (0.2 μmole)	0.290
DPNH + thyroxine (0.05 μmole) + 3,3'5'T_3 (0.5 μmole)	0.250

The decrease in the rate of oxidation of ferrocytochrome *c* with time was much more pronounced with the myeloperoxidase preparation than with the horseradish peroxidase preparation, as was found for the oxidation of epinephrine[1].

AKAZAWA AND CONN have reported that ferrocytochrome *c* is aerobically oxidized in the presence of peroxidase, Mn^{++} and certain phenols[3]. Ferrocytochrome *c*, however, was not oxidized by peroxidase in the presence of Mn^{++}, oxygen and thyroxine or estradiol under the conditions employed in Table I. The rate of oxidation of ferrocytochrome *c* was followed for 5 min only and therefore an oxidation following a lag period of greater length, as was observed with epinephrine cannot be excluded. If

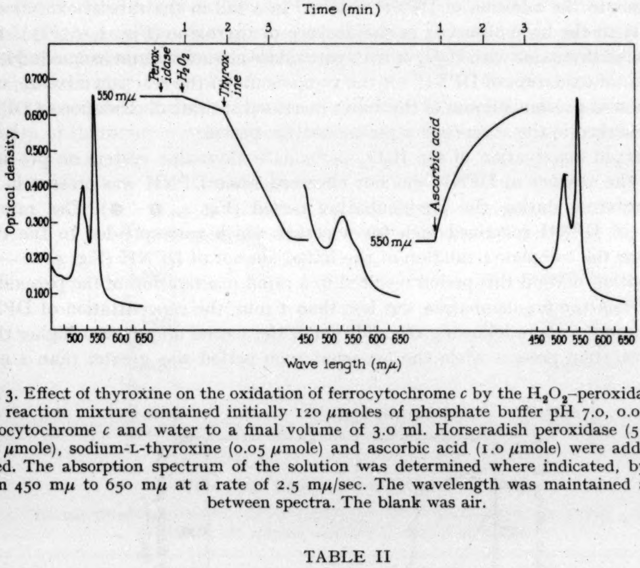

Fig. 3. Effect of thyroxine on the oxidation of ferrocytochrome *c* by the H_2O_2–peroxidase system. The reaction mixture contained initially 120 μmoles of phosphate buffer pH 7.0, 0.07 μmole of ferrocytochrome *c* and water to a final volume of 3.0 ml. Horseradish peroxidase (5 μg), H_2O_2 (1.0 μmole), sodium-L-thyroxine (0.05 μmole) and ascorbic acid (1.0 μmole) were added as indicated. The absorption spectrum of the solution was determined where indicated, by scanning from 450 mμ to 650 mμ at a rate of 2.5 mμ/sec. The wavelength was maintained at 550 mμ between spectra. The blank was air.

TABLE II

OXIDATION OF FERROCYTOCHROME *c* BY PEROXIDASE IN THE ABSENCE OF ADDED HYDROGEN PEROXIDE

The reaction mixture contained 120 μmoles of phosphate buffer pH 7.0, 0.07 μmole of ferrocytochrome *c* and water to a final volume of 3.0 ml. $MnCl_2$ (0.5 μmole), horseradish peroxidase 50 μg, DPNH (0.6 μmole), TPNH (0.6 μmole) and thyroxine (0.05 μmole) were added as indicated.

Supplements	*O.D. change/min at 550 mμ*
Mn^{++} + peroxidase	0.000
Mn^{++} + peroxidase + thyroxine	0.000
DPNH	0.000
Mn^{++} + peroxidase + DPNH	0.040
Mn^{++} + peroxidase + DPNH + thyroxine	0.256
TPNH	0.000
Mn^{++} + peroxidase + TPNH	0.040
Mn^{++} + peroxidase + TPNH + thyroxine	0.312

DPNH or TPNH also was added to the reaction mixture, the immediate oxidation of ferrocytochrome *c* was observed and this oxidation was greatly stimulated by the addition of thyroxine (Table I). Estradiol could replace thyroxine in this system but, as in the case of epinephrine oxidation, a considerably higher concentration of DPNH or TPNH was required. Horseradish peroxidase could be replaced by myeloperoxidase. Catalase inhibited the aerobic oxidation of ferrocytochrome *c* as was observed for the oxidation of epinephrine.

Inactivation of thyroxine

The pre-incubation of thyroxine with peroxidase and hydrogen peroxide for 15 sec prior to the addition of DPNH resulted in a fall in the rate of oxidation of the DPNH to the level observed in the absence of thyroxine (Fig. 4, ○—○). Pre-incubation of thyroxine with H_2O_2 or with peroxidase alone for 3 min had no influence on the rate of oxidation of DPNH. Of the components of the reaction mixture, only the addition of a second aliquot of thyroxine increased the rate of oxidation of DPNH to that observed in the absence of a pre-incubation period.

The rapid inactivation of the H_2O_2–peroxidase–thyroxine system on pre-incubation in the absence of DPNH was not observed when DPNH was present in the reaction mixture during the pre-incubation period (Fig. 4, ●—●). The rate of oxidation of DPNH remained high for a period which corresponded to the time required for the complete oxidation of the initial aliquot of DPNH (Fig. 4, ------). Pre-incubation beyond this period resulted in a rapid inactivation of the peroxidase system. When the pre-incubation was less than 1 min, the concentration of DPNH in the reaction mixture following the addition of the second aliquot was higher than the concentration present when the pre-incubation period was greater than 1 min.

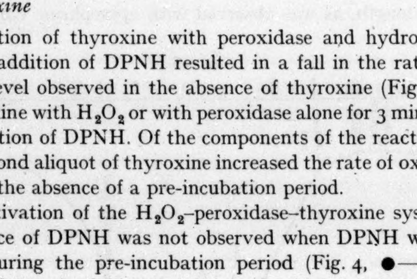

Fig. 4. The inactivation of thyroxine by peroxidase and hydrogen peroxide. ○—○, the reaction mixture which contained 120 μmoles of phosphate buffer pH 7.0, 0.05 μmole of thyroxine, 100 μg of horseradish peroxidase, 1.0 μmole of H_2O_2 and water to a final volume of 3.0 ml was pre-incubated at room temperature (28°) for the periods indicated. DPNH (0.3 μmole) was added at the end of the pre-incubation period except in the flask represented by zero pre-incubation in which case the DPNH was added prior to H_2O_2. The rate of oxidation of the DPNH was expressed as O.D. change per min at 340 mμ. ●—●, as above except that 0.3 μmole of DPNH was present in the reaction mixture at the beginning of the pre-incubation period. ------, the oxidation of the initial aliquot of DPNH is indicated by the fall in absorption at 340 mμ. A second aliquot of DPNH (0.3 μmole) was added following the pre-incubation of the reaction mixture for the periods indicated and the rate of oxidation of the DPNH was determined.

However, variation in the initial concentration of DPNH over wide limits did not affect its rate of oxidation[2].

These data indicate that the pre-incubation of thyroxine with H_2O_2 and peroxidase results in the oxidation of thyroxine to a form which is inactive as a stimulant of reactions catalyzed by peroxidase and that the presence of a suitable hydrogen donor in the reaction mixture protects thyroxine from this inactivation. DPNH was employed in Fig. 4 as the hydrogen donor. However, similar results were obtained with epinephrine.

In contrast to the results observed with the H_2O_2–peroxidase system (Fig. 4), the pre-incubation of thyroxine with Mn^{++}, peroxidase and oxygen for 4 min had little effect on the rate of oxidation of DPNH added at the end of the pre-incubation period (Fig. 5, ○—○). However, if DPNH was present in the reaction mixture during the pre-incubation period, a rapid inactivation of the peroxidase system occurred, following a lag period which corresponded to the time required for the complete oxidation of the initial aliquot of DPNH (Fig. 5, ●—●). Again, only the addition of thyroxine restored the peroxidase activity to the initial high levels. This would suggest that the oxidation of DPNH by peroxidase in the presence of Mn^{++}, oxygen and thyroxine results in the accumulation of an oxidant, possibly H_2O_2, in sufficient quantity to inactivate the thyroxine when the protective effect of DPNH is gone. AKAZAWA AND CONN could not demonstrate an accumulation of H_2O_2 during the aerobic oxidation of DPNH by horseradish peroxidase[3] while H_2O_2 does appear to accumulate during the aerobic oxidation of triose reductone by turnip peroxidase[8]. The oxidation of thyroxine and certain thyroxine analogues by H_2O_2 and peroxidase has been reported previously by MAYRARQUE-KODJA, BOUCHILLOUX AND LISSITZKY[9].

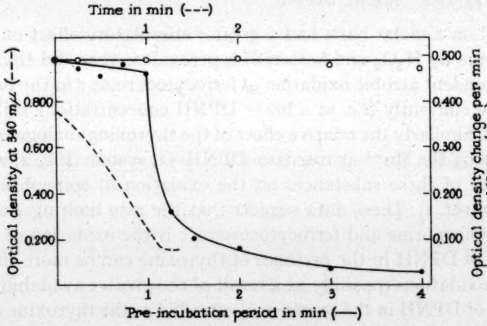

Fig. 5. The inactivation of thyroxine by peroxidase, Mn^{++} and oxygen. As in Fig. 4 except that Mn^{++} (0.5 μmole) was added instead of H_2O_2.

DISCUSSION

In the oxidation of DPNH by peroxidase in the presence of Mn^{++}, oxygen and certain phenolic compounds, 2 moles of DPNH are oxidized for every mole of oxygen taken up[2–4]. Catalase inhibits the oxidation of DPNH by this system[2–4] which suggests that H_2O_2 is formed and utilized during the course of the reaction. AKAZAWA AND CONN were unable to demonstrate the accumulation of H_2O_2 during the oxidation of DPNH by horseradish peroxidase in the presence of Mn^{++}, resorcinol and oxygen[3] which suggests that under these conditions the steady state concentration of H_2O_2 is very low. The process can be envisaged as follows:

$$\text{DPNH} + H^+ + O_2 \longrightarrow \text{DPN}^+ + H_2O_2 \tag{1}$$

$$\text{DPNH} + H^+ + H_2O_2 \longrightarrow \text{DPN}^+ + 2H_2O \tag{2}$$

$$2\text{DPNH} + 2H^+ + O_2 \longrightarrow 2\text{DPN}^+ + 2H_2O \tag{3}$$

Eqns. 1 and 2 have been simplified for the purpose of this discussion and represent overall reactions which also involve Mn^{++} and a phenolic compound. The inhibitory effect of catalase on the DPNH dependent oxidation of epinephrine and ferrocytochrome *c* by peroxidase suggests that H_2O_2 is an intermediate in these reactions. It is suggested therefore that the initial oxidation of DPNH by the peroxidase system generates an oxidant which is either H_2O_2 itself or is dependent on H_2O_2 for its formation and that this oxidant can be utilized in a secondary oxidation if the second substance being oxidized can successfully compete with DPNH for the H_2O_2 formed.

Estradiol on a molar basis had a greater stimulatory effect on the oxidation of ferrocytochrome by H_2O_2 and horseradish peroxidase than did thyroxine. However, the Mn^{++} dependent aerobic oxidation of ferrocytochrome *c* in the presence of DPNH occurred more efficiently (*i.e.* at a lower DPNH concentration) with thyroxine than with estradiol. Similarly the relative effect of the thyroxine analogues on the oxidation of epinephrine by the Mn^{++}–peroxidase–DPNH–O_2 system (Fig. 2) was very different from the effect of these substances on the oxidation of epinephrine by peroxidase and H_2O_2 (see ref. 1). These data suggest that the rate limiting step in the coupled oxidation of epinephrine and ferrocytochrome *c* is the oxidation of DPNH and that the oxidation of DPNH in the presence of thyroxine can be more effectively coupled to secondary oxidations (possibly as a result of the greater availability of H_2O_2) than the oxidation of DPNH in the presence of estradiol or the thyroxine analogues tested.

In certain instances (*e.g.* ferrocytochrome *c* and epinephrine oxidation) the secondary oxidation in the coupled system is sensitive to thyroxine and estradiol. However, this need not be the case for the overall reaction to be phenol sensitive since the rate limiting step in the coupled oxidation is the oxidation of DPNH and the latter reaction is stimulated by thyroxine and estradiol as well as by other phenols. In a previous study[10], estradiol was found to be inactivated by peroxidase, manganese and oxygen if DPNH (or TPNH) was present in the reaction mixture and a stimulation by thyroxine was evident when suboptimal concentrations of DPNH (or TPNH) were employed. The DPNH or TPNH dependent inactivation of estradiol by peroxidase is an instance in which the secondary oxidation, *i.e.* the inactivation of estradiol by H_2O_2 and peroxidase, does not appear to be thyroxine sensitive whereas the overall reaction is thyroxine sensitive[10]. Indeed according to this formulation, the secondary oxidation need not involve peroxidase at all but may involve catalase, or be nonenzymic and still be sensitive to thyroxine or estradiol. In this way the number of substances whose oxidation by peroxidase is associated with an oxygen uptake and is stimulated by thyroxine and estradiol as well as by certain other phenolic compounds would be increased considerably.

ACKNOWLEDGEMENTS

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REFERENCES

[1] S. J. KLEBANOFF, *J. Biol. Chem.*, 234 (1959) 2437.

[2] S. J. KLEBANOFF, *J. Biol. Chem.*, 234 (1959) 2480.

[3] T. AKAZAWA AND E. E. CONN, *J. Biol. Chem.*, 232 (1958) 403.

[4] H. G. WILLIAMS-ASHMAN, M. CASSMAN AND M. KLAVINS, *Nature*, 184 (1959) 427.

[5] A. P. MARTIN, H. A. NEUFELD, F. V. LUCAS AND E. J. STOTZ, *J. Biol. Chem.*, 233 (1958) 206.

[6] A. M. ALTSCHUL, R. ABRAMS AND T. R. HOGNESS, *J. Biol. Chem.*, 136 (1940) 777.

[7] B. CHANCE, in J. T. EDSALL, *Enzymes and Enzyme systems*, Harvard Univ. Press, Cambridge, 1951, p. 93.

[8] I. YAMAZAKI, K. FUJINAGA, I. TAKEHARA AND H. TAKAHASHI, *J. Biochem.* (*Tokyo*), 43 (1956) 377.

[9] A. MAYRARQUE-KODJA, S. BOUCHILLOUX AND S. LISSITZKY, *Bull. soc. chim. biol.*, 40 (1958) 815.

[10] S. J. KLEBANOFF AND S. J. SEGAL, *J. Biol. Chem.*, 235 (1960) 52.

THE SYNTHESIS OF THE INDUCED ENZYME, "CYANASE", IN *E. COLI*

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SUMMARY

An enzyme, cyanase, which breaks down cyanate to CO_2 and ammonia has been demonstrated in *E. coli*. The enzyme is adaptive and is synthesised in the lag as well as the logarithmic growth phase of the cells. Concentrations that inhibit the growth of *E. coli* permit enzyme synthesis in the lag phase but not in the logarithmic growth phase unless the preliminary growth of the cells occurs in low concentrations of cyanate. The enzyme has been partially purified, and its K_m value, pH sensitivity and stability have been described. The synthesis of the enzyme may be inhibited by various nucleic acid antagonists as well as by the inhibition of protein synthesis. Cyanase activity itself cannot be inhibited by KCN, NaF, and only the activity is depressed by KCNS while the yield remains unaffected. The effect of bacteriophage infection on the synthesis of the enzyme has been described, and it has also been shown that simultaneous synthesis of cyanase and β-galactosidase can take place.

INTRODUCTION

In a preliminary report it was stated[1] that addition of KCNO to the growth medium of *E. coli* prevents growth only for a period. It was also found that when *E. coli* was grown in the presence of low concentrations of cyanate, further addition of higher concentrations of KCNO did not inhibit the growth of the cells. In contrast, bacteria grown without addition of low concentrations of KCNO were inhibited by 0.02 *M* KCNO when it was added during the logarithmic growth phase. These findings suggested that an induced enzyme may be formed in the cells in the presence of KCNO and that this enzyme breaks down KCNO to a harmless product permitting cell growth. After an unusually long lag phase, the length of which depends on the concentrations of cyanate, the bacteria begin to grow.

The purpose of the experiments reported here was to confirm the presence of such an inducible enzyme, to characterize it and to determine the conditions permitting its synthesis.

Abbreviations: DEAE, diethyl aminoethyl; RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

METHODS AND MATERIALS

Organisms and growth conditions

E. coli B and B/1,5 and *B. megaterium* were maintained on nutrient agar slopes and were subcultured daily. The cultures were grown with aeration in Difco nutrient broth and the concentration of cells was determined turbidimetrically. The bacteriophage T_1 was prepared by the method of CREASER AND TAUSSIG[2] and assayed as described by ADAMS[3]. Cell free extracts of bacteria were prepared by shaking with glass beads. Protoplasts of *E. coli* were prepared using lysozyme and Versene as described earlier[4], and protoplasts from *B. megaterium* were prepared according to the method of WEIBULL[5]. 5 m*M* phosphate buffer, pH 7.0, containing 3 m*M* $CaCl_2$ was used to wash and suspend the cells.

Chemicals and determinations

All chemicals used were reagent grade. Carbamyl phosphate was prepared according to the method of JONES *et al.*[6], and assayed by its ability to convert ornithine to citrulline in the presence of ornithine transcarbamylase[6]. DEAE was prepared as described by PETERSON AND SOBER[7]. Ammonia was determined according to the method of BRAGANCA *et al.*[8]. β-galactosidase was measured by the method of LEDERBERG[9].

RESULTS

Cyanase determination

The induced enzyme, which will be referred to as cyanase, was determined manometrically. The principle of the determination was as follows: It was observed that when KCNO was incubated in the presence of the enzyme the medium became alkaline. When the incubation was carried out in the presence of a bicarbonate buffer anaerobically in conventional Warburg vessels using N_2–O_2 (95:5) as the gas phase, CO_2 was taken up. The CO_2 uptake was proportional to the substrate concentration. It can be seen in Table I that when the reaction was allowed to proceed to equilibrium, for each mole of KCNO about 0.75 mole of CO_2 was taken up. The results conform with the following reactions:

TABLE I

RELATION BETWEEN CYANATE CONCENTRATION AND CO_2 UPTAKE

Adapted bacterial extracts were the source of enzyme and the results were expressed as µmoles CO_2 uptake when the experiment was allowed to go to completion.

KCNO concentration	*µmoles CO_2 uptake*	*% theoretical*
20 µmoles	15.4	77
15 µmoles	11.5	77
10 µmoles	7.5	75

$$3H_2O + CNO^- \rightleftharpoons NH_4^+ + OH^- + HCO_3^- \quad (1)$$

$$OH^- + CO_2 \rightleftharpoons HCO_3^- \quad (2)$$

i.e. Total reaction =

$$3H_2O + KCNO + CO_2 \longrightarrow NH_4HCO_3 + KHCO_3 \quad (3)$$

i.e. in an atmosphere of CO_2 there is one mole of CO_2 taken up for each mole of KCNO decomposed.

Consequently, enzyme activity may be measured manometrically by measuring the rate of CO_2 uptake by the alkali produced by KCNO breakdown. There was no spontaneous breakdown of KCNO under the present incubation conditions.

The fact that there is no equivalence of KCNO decomposition and CO_2 uptake may be explained as follows: The alkalinity due to KCNO breakdown appears to be neutralized to about 75 % by the CO_2 in the atmosphere, while the rest is not neutralized but is reflected by a change in pH. When more bicarbonate buffer and more CO_2 in the atmosphere was used, the reaction went to a higher percentage of the theoretical and the shift in pH was also less. It is not due to the equilibrium of the reaction ($3H_2O + KCNO \longrightarrow NH_4HCO_3 + KOH$) since estimation of the ammonia produced indicated that when extracts of adapted cells were incubated with KCNO, the nitrogen was quantitatively converted to ammonia.

Production of cyanase

E. coli B/1 was grown in the presence and absence of 0.01 *M* KCNO in nutrient broth containing 1 mg/ml glucose to a concentration of about 10^9 cells/ml. After the cells were harvested they were resuspended in phosphate–calcium buffer and the cyanase activity was measured manometrically. It can be seen from Fig. 1 that cells which were grown in the presence of KCNO decomposed cyanate but not those grown in its absence.

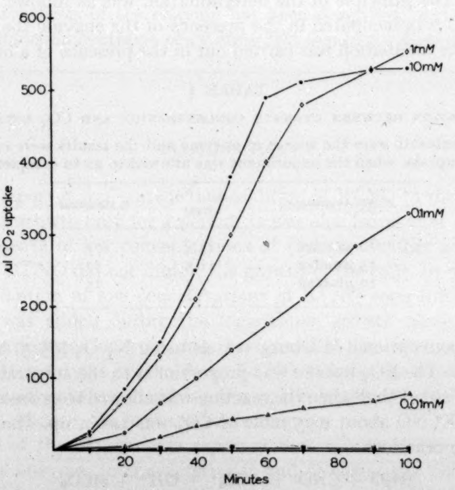

Fig. 1. Breakdown of KCNO by *E. coli* B/1,5 grown in the presence and absence of cyanate. Results expressed as µl CO_2 uptake from 30 µmoles KCNO by 10^{10} cells. Cells grown to 10^9/ml in nutrient broth plus 1 mg/ml glucose and ■—■, Nil; △—△, 0.01 m*M* KCNO; ○—○, 0.1 m*M* KCNO; ◇—◇, 1.0 m*M* KCNO; ●—●, 10.0 m*M* KCNO.

Purification and some properties of cyanase

Attempts to purify and concentrate the enzyme have been made by means of high speed centrifugation of cellular extracts and fractionation of the high speed supernatant on DEAE cellulose. The fractionation was carried out as described earlier[2].

Results of this purification may be seen in Table II.

TABLE II

PURIFICATION OF CYANASE

Fraction	*Enzyme activity/mg protein μmoles CO_2 uptake in 30 min/mg protein*
Crude extract	11
144,000 × *g* supernatant	21
Sediment	0
DEAE: not absorbed	0
0.1 *M* NaCl	0
0.2 *M* NaCl	38
0.3 *M* NaCl	31
0.4 *M* NaCl	2.4
0.5 *M* NaCl	0

The 0.2 *M* NaCl eluted fraction was considered sufficiently pure to determine the K_m value of the enzyme, and Fig. 2 shows that the K_m value for cyanase is $2.9 \cdot 10^{-2}$ *M*. The pH sensitivity of the enzyme is not very great. Although the optimal pH seems to be around 7, activity is quite high at pH 6 as well as at pH 8. The extract keeps well in the frozen state at —20° for weeks, but 1 min boiling destroys the activity completely. Dialysis against distilled water has no effect on the activity of cyanase.

Induction of enzyme synthesis related to growth cycle of bacteria

Experiments were performed to establish the phase of the growth cycle of *E. coli* when enzyme synthesis occurs.

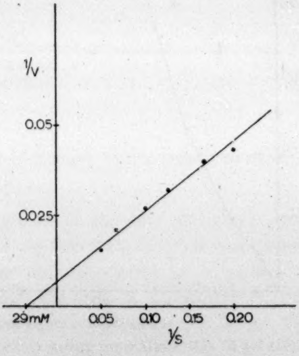

Fig. 2. The effect of substrate concentration on the velocity of cyanate decomposition. 0.2 *M* NaCl eluate from DEAE was used as source of enzyme. The rates are measured as described in text using μl CO_2 uptake in 10 min.

1.5 l of nutrient broth were inoculated with 2-day old *E. coli* B/1,5 slant so that the final concentration of cells was $2.5 \cdot 10^8$ cells/ml. Samples were taken at 30-, 60-, and 90-min intervals. The bacteria were centrifuged, washed, resuspended in phosphate–calcium buffer, and cyanase activity was determined.

The cyanase activity expressed as μmoles CO_2 uptake in 30 min brought about by 10^{11} cells exposed to KCNO for varying periods is shown in Table III.

TABLE III

CYANASE SYNTHESIS IN THE LAG PHASE OF GROWTH BY *E. coli* B/1,5

Bacteria were incubated in presence of 10 m*M* KCNO for the stated time. Activity is expressed as μl CO_2 uptake in 10 min by 30 μmoles KCNO in presence of 10^{11} cells.

Time of exposure to KCNO minutes	*0*	*30*	*60*	*90*
Cell count/ml	$3 \cdot 10^8$	$3.1 \cdot 10^8$	$3.3 \cdot 10^8$	$5 \cdot 10^8$
Cyanase activity	0	102	152	360

It can be seen from Table III that synthesis of cyanase takes place before the bacteria reach the logarithmic growth phase, although it cannot be unequivocally stated that no growth took place during this time.

The rate of cyanase synthesis was determined by allowing 1 m*M* KCNO to be present in cultures of bacteria growing logarithmically for various times. The results of this experiment are shown in Fig. 3. It can be seen that synthesis of cyanase begins immediately and the maximum amount is reached somewhere between 15 and 30 min.

Concentration of substrate necessary for induction

It was also of interest to find out the minimum amount of KCNO that will induce enzyme formation and the maximum amount that will permit cyanase synthesis.

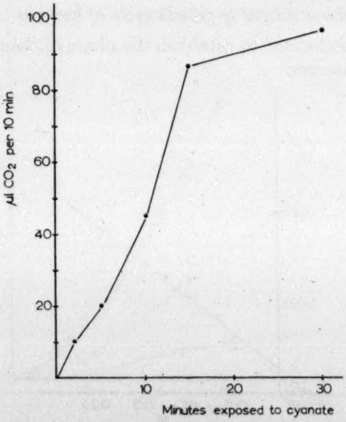

Fig. 3. Rate of cyanase synthesis by *E. coli*. Cells were grown in nutrient broth-glucose to about $5 \cdot 10^8$ cells/ml. KCNO was then added at a concentration of 1 m*M* for increasing periods. Samples were taken at these times, washed and resuspended to a concentration of $1.5 \cdot 10^{10}$ cells/3 ml containing 15 μmoles KCNO.

The results of this experiment are shown in Table IV. It can be seen that 0.01 m*M* KCNO is sufficient to induce synthesis of enzyme and that addition of 20 m*M* KCNO does not permit enzyme synthesis. In all these cases KCNO was added to logarithmically growing *E. coli* B/1,5 and was allowed to act for 15 min. However, it is also shown in Table IV that if the substrate is allowed to be present for not a predetermined period but for as long as the bacteria reach a given cell concentration, the results are strikingly different, suggesting that exhaustion of the substrate results in cessation of further synthesis and consequent dilution of the enzyme occurs. This was further confirmed in an experiment where exposure of *E. coli* to KCNO for various times was followed by removal of KCNO by centrifugation of the cells and resuspension and further growth of the cells in fresh nutrient broth. The results shown in Table V, expressed as enzyme activity per cell, indicate that enzyme activity drops if cells are reincubated in the absence of substrate.

TABLE IV

CYANASE SYNTHESIS IN THE PRESENCE OF VARIOUS AMOUNTS OF KCNO

Bacteria (a) exposed to KCNO for 15 min, or (b) grown in the presence of the stated amounts of KCNO to 10^9 cells/ml. Activity: μl CO_2 uptake from 30 μmoles KCNO in 30 min per 10^{10} cells.

*KCNO m*M	*0*	*0.01*	*0.1*	*1.0*	*10*	*20*
(a)	0	75	375	258	18	0
(b)	0	22	63	130	155	160

TABLE V

EFFECT OF LENGTH OF EXPOSURE TO CYANATE ON ENZYME SYNTHESIS

Expressed as μl CO_2 uptake in 30 min per 10^{10} cells from 30 μmoles KCNO

Time of exposure to KCNO minutes	*0*	*5*	*10*	*15*
Tested immediately	0	145	276	420
Reincubated 4 hours in absence of substrate	0	56	90	76

Effect of low concentrations of cyanate in the growth medium on enzyme synthesis in the logarithmic growth phase

E. coli B/1,5 was grown in the presence and absence of 1 m*M* KCNO to a concentration of $5 \cdot 10^8$ cells/ml. At this time cyanate was added to a final concentration of 20 m*M* and the bacteria were incubated for a further 20 min. Table VI demonstrates the level of enzyme in the cells at the beginning and end of the experiment as well as the growth of the cells under these conditions, and it can be seen that the cells which were grown in the presence of KCNO were able to grow and synthesize further amounts of the enzyme, while those grown in the absence of cyanate could neither grow nor could they be induced.

TABLE VI

EFFECT OF CYANATE IN THE GROWTH MEDIUM ON SUBSEQUENT CYANASE SYNTHESIS

Cyanate concentration in growth medium	*Amount of enzyme**		*E. coli/ml*		*Factor of increase in growth*
	at 0 time	*at 20 min*	*0 min*	*20 min*	
1 m*M*	6	12	5.9×10^8	7.8×10^8	1.32
Nil	0	0	6.8×10^8	7.1×10^8	1.04

* Amount of enzyme expressed as μmoles CO_2 uptake per 10^{10} cells from 30 μmoles KCNO in 30 min.

Inhibition of enzyme synthesis

In order to gain an insight into the cellular mechanisms necessary for the synthesis of cyanase, various substances that are known to have an effect on protein or nucleic acid synthesis or on the metabolism of the cells were tested for their effect on cyanase production. Table VII illustrates the effect of these substances. Cyanase activity is expressed as μl CO_2 uptake by 10^{10} cells from 30 μmoles KCNO in 30 min. It can be seen from Table VII that chloramphenicol and KCN inhibit the synthesis of the enzyme but the other substances produce complete inhibition only if added prior to the substrate. When the bacteria were grown aerobically and induction took place under anaerobic conditions, or when growth as well as induction took place anaerobically, there was no noticeable diminution in the level of enzyme activity.

TABLE VII

EFFECT OF VARIOUS SUBSTANCES ON CYANASE SYNTHESIS

Substance	*Time of KCNO addition*	*μl CO_2/30 min per 10^{10} cells*	*% inhibition*
Nil	0	485	0
100 μg streptomycin	0	286	41
100 μg streptomycin	30	15	97
20 μg chloramphenicol	0	0	100
20 μg chloramphenicol	30	0	100
10 μg vancomycin	0	384	21
10 μg vancomycin	30	100	80
2 μg mitomycin	0	420	13
2 μg mitomycin	30	160	67
20 μg mitomycin	0	300	38
20 μg mitomycin	30	0	100
100 μg 8-azaguanine	0	480	2
100 μg 8-azaguanine	30	80	84
100 μg 5-bromouracil	0	490	0
100 μg 5-bromouracil	30	68	86
100 μg 5-nitroorotic acid	0	495	0
100 μg 5-nitroorotic acid	30	130	73
100 μg 2-4-dichloropyrimidine	0	400	18
100 μg 2-4-dichloropyrimidine	30	120	75
0.01 *M* KCN	0	25	95
0.01 *M* KCN	30	25	95

1 m*M* KCNO was added to each sample at indicated time and was allowed to be present for 30 min.

The effect of phage on enzyme synthesis

The effect of phage infection on cyanase synthesis is demonstrated in Table VIII. It can be seen in Table VIII that infection with T_1 prevents adaptive enzyme synthesis to a large extent even if added together with the inducer and that there is complete inhibition of synthesis if infection precedes induction. However, Table VIII also shows that while the infection may reduce the amount of enzyme in the cells, if virus is allowed to be present for longer periods it does not abolish the already existing enzyme.

TABLE VIII

EFFECT OF BACTERIOPHAGE T_1 ON CYANASE SYNTHESIS

E. coli B was grown in nutrient broth-glucose to $5 \cdot 10^8$ cells/ml. 0.1 μmoles/ml KCNO was added for 15 min, except when stated differently, then T_1 was added for the stated times in a 10-fold excess. Results expressed as μl CO_2 per $2 \cdot 10^{10}$ cells per 30 min released from 30 μmoles KCNO.

Conditions	*μl CO_2*
No virus, 1 m*M* KCNO added at 0 time (for 10 min)	210
T_1 and 1 m*M* KCNO added at 0 time (for 10 min)	45
T_1 at 0 time, 1 m*M* KCNO 5 min later (KCNO for 10 min)	0
Time of exposure to particles	
0	580
5	570
8	540
10	420
12	309
Lysate	360

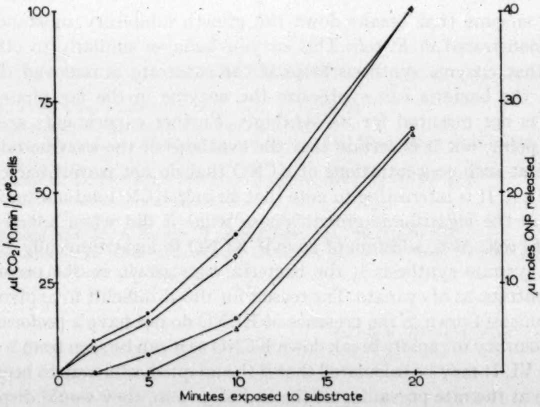

Fig. 4. Simultaneous induction of cyanase and β-galactosidase synthesis in *E. coli*. Cells were grown in nutrient broth and 1 mg/ml glucose to a concentration of $5 \cdot 10^8$ cells/ml. Then flasks were set up to contain 5 mg/ml lactose and/or 1 μmole/ml KCNO for varying periods. Samples were centrifuged washed and resuspended in the appropriate buffer for determinations. Results expressed as μl CO_2 uptake from 30 μmoles KCNO per 10^{10} cells and as μmoles *o*-nitrophenol released from ONPG by 10^9 cells. The cells were exposed to ●—●, 1 m*M* KCNO, and ◇—◇, 1 m*M* KCNO + 5 mg/ml lactose: cyanase activity; ○—○, 5 mg/ml lactose and △—△, 5 mg/ml lactose + 1 m*M* KCNO: β-galactosidase activity.

Simultaneous induction to two substrates

It was of interest to investigate the effect of the simultaneous induction by another substrate, such as lactose, to induce β-galactosidase, on the synthesis of cyanase. Fig. 4 demonstrates that simultaneous exposure to lactose and KCNO has no adverse effect on either of the two enzyme systems and it can be seen that both enzymes are synthesised at a rapid rate.

Specificity

Attempts were made to induce cyanase synthesis by KCNS, but it was not successful. Carbamyl phosphate acted as an inducer; however, this may be due to the fact that low concentrations of KCNO are always present when carbamyl phosphate is added to the growth medium and these low concentrations may be sufficient to induce cyanase synthesis. Cyanase was found to break down carbamyl phosphate although somewhat slower than it broke down cyanate, but had no effect on KCNS.

However, KCNS did reduce cyanase activity and the reduction was proportionate to the KCNS concentration, but the final amounts of both CO_2 uptake and ammonia released from KCNO breakdown were the same. KCN or NaF inhibited neither the rate of cyanate breakdown nor the final amount of KCNO broken down.

Attempts were also made to induce cyanase synthesis in *B. megaterium*, the protoplasts prepared from *B. megaterium* and protoplasts prepared from *E. coli*. In none of these systems was there any evidence of cyanase production.

DISCUSSION

An adaptive enzyme that breaks down the growth inhibitory substance, cyanate, has been demonstrated in *E. coli*. This enzyme behaves similarly to other induced enzymes in that enzyme synthesis stops if the substrate is removed. However, it appears that the bacteria can synthesize the enzyme in the lag phase suggesting that growth is not essential for its synthesis. Further experiments are needed to confirm this point, but it is certain that the synthesis of the enzyme takes place in the lag phase at such concentrations of KCNO that do not permit bacterial growth for as long as 4 h. It is interesting to note that 20 m*M* KCNO did not induce cyanase when added in the logarithmic growth phase while it did when added during the lag phase of *E. coli*. Also, addition of 20 m*M* KCNO to logarithmically growing cells *will* produce cyanase synthesis if the bacteria were grown in the presence of low (1 m*M*) concentrations of cyanate. The reason for this is difficult to explain especially since the organisms grown in the presence of KCNO do not have a preformed enzyme in sufficient quantity to rapidly break down KCNO as it can be seen from b of Table IV or from Table VI. It may be calculated that if the adapted cells were to begin breaking down cyanate at the rate prevailing at the end of 30 min, they would dispose of only about 0.6 μmole of KCNO/30 min from the growth medium still leaving 19.4 μmoles KCNO/ml. These cells can also grow in the presence of 20 m*M* KCNO in contrast to organisms grown in the absence of low concentrations of cyanate.

The rate of synthesis has been found comparable with the rate of synthesis of β-galactosidase, used as an example of another induced enzyme. The concentration of KCNO necessary to bring about induction is quite low, 0.01 m*M* KCNO being sufficient. Optimal enzyme synthesis occurs in the logarithmic growth phase at concentrations which do not inhibit growth.

The results obtained with the few inhibitors are not unusual. The various structural analogues of purines and pyrimidines were examined in the hope that it would be possible to show the need for either RNA or DNA only for enzyme synthesis. However, it can be seen in Table VI that all the substances tested inhibited to some degree the synthesis of cyanase. It is not surprising that the substances which influence nucleic acid caused complete inhibition of enzyme only if added prior to the inducer, since the synthesis of cyanase begins immediately and it would go on until normal nucleic acid was available. Since it would take some 10 min for, *e.g.*, 8-azaguanine to exert its effect on nucleic acids[10], it is natural that for 10 min or so cyanase synthesis should go on unhindered. In that period, a near maximum amount of cyanase would be synthesized. In the case of the effect of T_1 infection, it is clear that infection before induction will prevent induction, while the results shown in Table VIII suggest that there is no effect on the already formed enzyme until 10–12 min after infection, at which time the enzyme begins to decrease because it is being used for virus protein synthesis. Unfortunately, at present the objection that perhaps at later stages of the infection some lysis of the bacteria results in dilution of the enzyme cannot be answered, although every precaution has been taken in the experiments to prevent this and the results shown were typical.

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REFERENCES

[1] A. TAUSSIG, *Proc. Can. Federation Biol. Soc.*, 2 (1959) 64.
[2] E. H. CREASER AND A. TAUSSIG, *Virology*, 4 (1957) 200.
[3] M. H. ADAMS, *Methods in Medical Research*, Vol. II, Year Book Publishers, Inc., Chicago, 1950, p. 1–73.
[4] P. ROGERS AND G. D. NOVELLI, *Biochim. Biophys. Acta*, 33 (1959) 423.
[5] C. WEIBULL, *J. Bacteriol.*, 66 (1953) 688.
[6] M. E. JONES, L. SPECTOR AND F. LIPMAN, *J. Am. Chem. Soc.*, 77 (1955) 819.
[7] E. A. PETERSON AND H. A. SOBER, *J. Am. Chem. Soc.*, 78 (1956) 751.
[8] B. M. BRAGANCA, J. H. QUASTEL AND R. SHUCHER, *Arch. Biochem. Biophys.*, 52 (1954) 18.
[9] J. LEDERBERG, *J. Bacteriol.*, 60 (1950) 381.
[10] E. H. CREASER, *Biochem. J.*, 64 (1956) 539.

NITROGEN FIXATION IN CELL-FREE EXTRACTS OF *CLOSTRIDIUM PASTEURIANUM*

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SUMMARY

Reproducible nitrogen fixation has been achieved in cell-free enzyme preparations. Extracts of *Clostridium pasteurianum* underwent enrichments averaging 0.7 atom % excess ^{15}N when exposed to 60 atom % excess $^{15}N_2$ for 1 h. Activity was not sedimented at 144,000 × *g* in 4 h, demonstrating that the nitrogenase is non-particulate.

Several experimental conditions were critical in securing active extracts. These included: (a) use of particular cell-breaking methods, (b) addition of an energy source, preferably sodium pyruvate, (c) close control of pH, and (d) strict exclusion of O_2. Consistently active preparations were obtained by disrupting frozen cells in a Hughes press or by autolysis of specially dried cells resuspended in phosphate buffer.

Nitrogen fixation in *C. pasteurianum* involves reduction to ammonia, since the $^{15}N_2$ taken up by the extracts could be recovered quantitatively as $^{15}NH_3$. Coupling of fixation to the clostridial-type phosphoroclastic reaction is inferred. Differential spectral responses at 300–365 mμ in the presence and absence of N_2 are observed and may represent interaction of nitrogen with the enzyme system.

INTRODUCTION

Progress in establishing the mechanism of biological nitrogen fixation has been slow because the enzyme system has appeared too labile for study outside the cell[1–6]. Existing information has been obtained largely from studies with living organisms. The end-product has been generally conceded to be ammonia[7–10], though there are differences of opinion about its pathway of formation[10–12]. The immediate source of hydrogen for the reduction is obscure[13], particularly in view of the inhibitory effects of molecular hydrogen[14–16]. Substances recognized to play some part in nitrogen fixation in growing cells include molybdenum[17], iron[18–19], and biotin[19].

In this report we describe methods for extracting and activating the nitrogen-fixing enzyme system from *Clostridium pasteurianum*. Availability of active extracts

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Abbreviations: CoA, coenzyme A; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; ATP, adenosine triphosphate.

* Contribution Number 619.

now opens the way for investigating the mechanism of nitrogen fixation at the enzyme level, and first results are presented. A preliminary note on this work has been published[20]. Details were communicated to Professors R. H. BURRIS and P. W. WILSON, who with their coworkers have confirmed the major findings and have extended the methods successfully to *Rhodospirillum rubrum*[21].

EXPERIMENTAL

Culture methods

Cultures of *C. pasteurianum* strain W-5 were obtained from the American Type Culture Collection (Catalog 6013), Washington, D.C., and grown under nitrogen-fixing conditions as described previously[19] except that the iron was added as alcoholic $FeCl_3$ solution. 2 ml of stock solution (5.0 g $FeCl_3 \cdot 6H_2O$ in 100 ml absolute ethanol) was used per liter of culture medium. Cultures with a generation time of 3 h or less were produced. Various size cultures were used: 0.5 l, 8 l, and 40 l in glass vessels (12-gallon solution bottles, A. H. Thomas Co., for the 40-l cultures). The inoculum was maintained as 500-ml cultures transferred daily. After about one hundred serial transfers, a fresh inoculum was started as previously described[19]. A 2% inoculum was used in each instance. After 16 h growth, the O.D. usually was 0.7–0.8 (650 mμ in a standard Klett tube in a "Lumetron" colorimeter), and the cultures were generally found to be at pH 5.4–5.8. The yield of cells after being washed and dried in a "Rinco" evaporator as described below was 0.63–0.75 g/l. During growth N_2 was bubbled through the cultures at 10–30 l/h to provide agitation and maintain anaerobiosis.

Buffer

Unless otherwise stated, the buffer employed throughout this work was 0.05 *M* potassium phosphate, pH 6.8, prepared by dissolving 6.80 g (0.05 mole) KH_2PO_4 in 400 ml distilled water, adjusting the pH to 6.8 with KOH, and diluting to 1 l.

Resting cells

For resting-cell experiments, 400 ml of a culture was centrifuged at 3,000 × *g* for 15 min. The sedimented cells were washed in 50 ml of buffer, recentrifuged, and suspended in 25 ml of buffer. Exclusion of air and maintenance of low temperatures were not essential during these steps. Fixation experiments were made in the absence of O_2.

Preparation of nitrogen-fixing extracts

Hughes press: The cells from 400 ml of a culture at O.D. 0.7–0.8 (650 mμ in a standard Klett tube in a "Lumetron" colorimeter) were sedimented at 27,000 × *g* for 15 min at 5°. The paste was washed with 25 ml of cold buffer, centrifuged, and resuspended in 9 ml of cold buffer. The suspension was poured into a Hughes press[22] previously cooled to — 35°. The piston was inserted, and after 5 min the frozen material was driven through the press by application of a Carver press or by 20–25 blows with a 4.5-kg weight dropped 2.4 m through a pipe guide. The press was opened in air, and the frozen preparation was rapidly removed without thawing. It was either transferred directly to a flask for assay of its nitrogen-fixing activity or was thawed under N_2 and centrifuged to remove debris and intact cells. Centrifugation was at 27,000 × *g* at 0° in a "Servall" refrigerated centrifuge for 15 min. The activity was in the cell-free supernatant solution and required protection from O_2.

Autolysis of dried cells: 10 l of a culture at O.D. 0.7–0.8 (650 mμ in a standard Klett tube in a "Lumetron" colorimeter) was centrifuged at 5–10° in a Sharpless supercentrifuge with N_2 pressure being used to force the liquid from the culture bottle into the centrifuge bowl. The cell paste was washed by suspension in 500 ml of cold buffer and recentrifuged. Enough distilled water, usually about 5–20 ml, at 5° was added to the cell paste so that the paste could be poured into a 5-l round-bottomed flask. The flask was attached to a "Rinco" rotating evaporator and the cells were dried *in vacuo* without freezing in a water bath at 30–40°. After 1 to 2 h the pressure had dropped to 0.5 to 0.25 mm. The flask was left *in vacuo* overnight without rotation. The dry cells, which weighed 6.3–7.5 g, were stored in sealed bottles at — 15° (see below).

10 g of dried cells were pulverized in a mortar and added to 100 ml of buffer in a 250-ml suction flask. Air was deleterious beyond this point. The flask was thoroughly purged with H_2, sealed with 0.8 atm of H_2 and placed on a Brunswick rotary shaker at 30° for 45 min. It was then cooled to 0° in an ice bath, and the contents were immediately poured into 50-ml centrifuge cups for centrifugation at 27,000 × *g* for 15 min at 0°. The supernatant solution was the cell-free nitrogen-fixing preparation.

Ultracentrifugation of extracts: When more rigorous centrifugation of the nitrogen-fixing preparations was desired, it was performed at 144,000 × *g* in a "Spinco" model L preparative ultracentrifuge for 3 to 4 h at 0°. All activity remained in solution.

Storage: The extracts were reasonably stable to storage provided they were kept under H_2 or N_2 and below 5°. 24-h storage under these conditions resulted in 20–50 % loss in activity. Storage beyond a few days resulted in total loss of activity. Freezing the extracts gave some improvement in storage life.

Dried cells prior to autolysis retained their full capacity to yield nitrogen-fixing extracts for over 2 months under air at — 15°, provided moisture was excluded. In the presence of moisture, air was rapidly destructive.

Assay for nitrogen-fixing activity

Extracts were assayed for nitrogen-fixing activity by adding sodium pyruvate and exposing to $^{15}N_2$, which was prepared and handled by the methods of BURRIS AND WILSON[23]. The proportions of extract, buffer, and sodium pyruvate were chosen so that the solution contained 10–20 mg of protein-nitrogen (biuret method) and 100–200 mg of sodium pyruvate in a total volume of 5–8 ml. Typical examples are presented in Tables IV, VI, and VII.

The sodium pyruvate (100 mg) and buffer (4 ml) were placed in a 25-ml round-bottomed flask, frozen, and then the extract (4 ml) was added. Purging with A was begun immediately. The flask was alternately evacuated and filled with A until thawing was completed and all entrapped air was released. At least five purgings were made. Finally, the flask was filled to 0.5 atm with 60 % enriched $^{15}N_2$, sealed, and agitated for 40–60 min at 30° on a "Gyrotory Shaker" (New Brunswick Scientific Co., New Brunswick, N.J.). Considerable CO_2 and H_2 accumulated in the flask during reaction. The solution was digested by Kjeldahl procedure[23], and the resulting NH_3 was converted to N_2 by the procedures of BURRIS AND WILSON[23]. The Fredericks

Modification[24] of the Kjeldahl procedure gave identical results. Dumas combustion of the lyophilized extracts produced erratic results probably due to the difficulty in combustion.

The $^{15}N/^{14}N$ ratio was determined with a Type 21-103C mass spectrometer (Consolidated Engineering Co., Pasadena, Calif., U.S.A.).

Spectral differentiation procedure

Difference spectra of the cell-free nitrogen-fixing extracts were obtained with the aid of the apparatus shown in Fig. 1. The "capillary bleed" in Fig. 1 was a capillary tube which was attached by a rubber sleeve to the tube at the mouth of the 25-ml flask, and it projected through the stopcock leading to the spectrophotometer cell. The capillary was snapped when this stopcock was closed to seal the cell just before transferring it to the spectrophotometer. Four of these assemblies were connected to a vacuum manifold which was supplied with H_2, N_2, and A, each scrubbed with aqueous chromous chloride. These gases analyzed less than 2 ppm O_2 (continuous flow O_2 amperometric analyzer, Lockwood and Mc Lorie, Inc., Hatboro, Pa., U.S.A.). In a typical experiment 3.5 ml of buffer containing 160 mg sodium pyruvate was placed in each 25-ml flask and degassed by stirring *in vacuo* at room temperature for 5 min. The flasks were then filled with H_2 and cooled to 5°. The flasks were opened momentarily and 0.5 ml of a nitrogen-fixing extract, centrifuged at 100,000 × *g* for 15 min and typically containing 4-6 mg protein-N/ml, was added. The flasks were evacuated, then filled and flushed with H_2 six times. Evacuations were conducted with the stopcock turned so that access to the flask was through the larger tubing (Fig. 1). The contents of the flasks were gently stirred and warmed to 25° for 15 min and again cooled to 5°. With the stirrer stopped, each flask was flushed twice and filled with the appropriate gas. The contents of each flask were again gently stirred at 5° for 15 min. The flasks were then inverted in an ice bath and the contents transferred into the spectrophotometer cell by application of reduced pressure (3-5 mm) through the capillary bleed. The spectrophotometer cell was sealed by closing its stopcock snapping the "capillary bleed" tube in the process. The cell together with its stopcock was detached as a unit from the rest of the assembly and transferred to the spectrophotometer without exposing the sample to air. The difference spectrum was obtained by using the sample under H_2 as the blank in a Beckman Model DU Spectrophotometer.

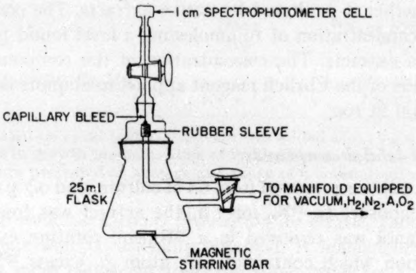
1 cm SPECTROPHOTOMETER CELL
CAPILLARY BLEED
RUBBER SLEEVE
25 ml FLASK
TO MANIFOLD EQUIPPED FOR VACUUM, H₂, N₂, A, O₂
MAGNETIC STIRRING BAR

Fig. 1. Spectral differentiation apparatus.

Tests for fixation intermediates

Experiments seeking evidence of fixation intermediates were concerned chiefly with hydrazine, hydroxylamine, nitrite, and dihydropyridazinone-5-carboxylic acid[11].

A cell-free extract (37 ml) was prepared from cells from 3 l of culture, and 300 mg of sodium pyruvate and 113 mg of $(NH_4)_2SO_4$ were added. The $(NH_4)_2SO_4$ was intended to repress ammonia synthesis and force accumulation of precursors. The solution was placed under N_2 and agitated at 30° with a magnetic stirrer. A control was assayed with $^{15}N_2$ to assure that fixation was occurring. Samples (5 ml) were withdrawn at intervals and freed of protein by treatment with 70% ethanol. Tests for hydrazine were made with Ehrlich's reagent[25]. Tests for hydroxylamine and nitrite were by the method of ENDRES AND KAUFMANN[26].

Evidence for involvement of dihydropyridazinone-5-carboxylic acid was sought by determining whether it is altered by active extracts. The compound was added to an extract at a concentration of 10 μmoles/ml, a level found to be noninhibitory to fixation in these extracts. The concentration of the compound[11] was measured at intervals by means of the Ehrlich reagent applied to aliquots before and after acid hydrolysis for 30 min at 100°.

Fractionation of ^{15}N-labeled compounds

An extract (60 ml) was prepared from 6 l of culture, and 0.7 g of sodium pyruvate was added. After exposure to $^{15}N_2$ for 1 h, the extract was freed of proteins with 70% ethanol. Ethanol was removed in a "Rinco" rotating evaporator at 2 mm pressure. The solution which contained 4.93 atom % excess ^{15}N and 16 mg total nitrogen was introduced to a 55 × 2.5 cm column of "Dowex" 50 resin. The compounds on the column were fractionated by elution with increasing concentrations of HCl (see ref. 27). Elution was followed by u.v. absorption measurements and by ninhydrin reaction. Initial identification was made by paper chromatography with butanol–acetic acid–water (1:3:4), acetone–urea–water (60:0.5:40), butanol–urea–water (60:0.5:40) and lutidine–water[28].

RESULTS AND DISCUSSION

Search for consistently active nitrogen-fixing extracts

The choice of *C. pasteurianum* for this work was based largely on the facts that it is an anaerobe and its nitrogen-fixing activity is relatively insensitive to hydrogen[15].

The design of experiments on enzyme extraction was aided by first establishing conditions for nitrogen fixation in resting cells. With the $^{15}N_2$ technique[23], cell suspensions of *C. pasteurianum* were found not to fix nitrogen unless supplied with a substrate, *e.g.*, sucrose or, better, pyruvate. Supplementary iron, molybdenum, and biotin, substances needed for fixation in growing cells, were not beneficial. The amount of fixation obtained relative to the initial nitrogen content was approximately 1 to 2% measured by the $^{15}N_2$ method and 2 to 5% in other experiments in which the less precise Kjeldahl method was used. Typical experiments are presented in Table I.

On the basis of the foregoing results, pyruvate was used in conjunction with the $^{15}N_2$ assay to investigate enzyme extraction methods.

Of a variety of cell-breaking techniques examined, only two have consistently given active nitrogen-fixing extracts; these are the Hughes press and the autolysis of dried cells. With 60 atom % excess $^{15}N_2$, extracts from either procedure gave enrichments averaging 0.7 atom % ^{15}N which corresponds to 12 μg N fixed/mg protein-N initially present. In the absence of pyruvate, no fixation occurred. The Hughes press method is simpler to control, but the autolysis method is less laborious and affords a larger capacity. Both are highly reproducible with due care to operating variables. Details are discussed in other sections.

TABLE I

NITROGEN FIXATION IN RESTING CELLS OF *C. pasteurianum*

*Additions**	*Percent nitrogen increase*	*Analytical method***
None	None	$^{15}N_2$ or Kjehdahl
Pyruvate	1.9	$^{15}N_2$
Pyruvate	4.5 ± 1.1	Kjeldahl
Pyruvate, biotin, Fe, Mo	4.4 ± 1.0	Kjeldahl
Sucrose	2.0 ± 1.0	Kjeldahl

* Cells were suspended in 25 ml buffer, pyruvate was added as 0.25 g sodium salt, and other concentrations were as in growth medium. Time of incubation was 1 h.

** Kjeldahl results are presented as average and range of 6 determinations.

Disruption with sonic oscillations proved highly erratic, though enrichments of 0.35 % in ^{15}N content were occasionally obtained. A homogenizer[29] operating with powdered glass was more consistent than the sonic oscillator though less effective than the Hughes press in both the fraction of cells disrupted and activity levels obtained. Little or no success was had in a limited number of experiments with acetone-drying, enzymic lysis, detergent lysis, freezing, or pressure changes.

Evidence for cell-free state of active extracts

That the nitrogen-fixing activity of these extracts was not due to intact cells or large particles was demonstrated by centrifugation. Supernatant fractions retained full activity and contained no visible particles by microscopic examination after 2 to 4 h at 144,000 × *g* (Table II). Activity was not lost in a series of centrifugations first at 27,000 × *g* and then at 144,000 × *g*. In each step only a small upper portion of the preceding supernatant liquid was taken by pipette to avoid resuspension of sedimented cells. Centrifugation seemed to enhance both fixation activity (Table II) and efficiency of pyruvate utilization (Table VI) in the extracts, and it was adopted as routine procedure (27,000 × *g* for 15 min).

Broken-cell preparations before centrifugation contained intact cells ranging from 0.7 % to 10 % but usually 2 to 5 % of the original population. Although some of the cells were viable as shown by culture experiments, they contributed little nitrogen-fixing activity. Thus, extracts containing 2 to 5 % of their original cells were up to seven times as active as their intact-cell controls (Table II).

General properties of the enzyme extracts

The optimum hydrogen ion concentration for fixation activity was pH 6.3–6.5 (Fig. 2). On either side of this range, activity decreased sharply. Inactivation in the range pH 5.5–6.3 could be reversed by readjusting to pH 6.3–6.8, but, after inactivation below pH 5.5, the system was not reactivated by pH adjustments.

TABLE II

NONDEPENDENCE OF ACTIVITY ON INTACT CELLS IN EXTRACTS

Hughes press method. 1 and 2 were from one preparation, 3 and 4 were from another. Intact-cell content is given relative to initial count. % ^{15}N enrichment = % ^{15}N final minus 0.36. Time of incubation was 1 h.

System and treatment	*Content intact-cells*	*% ^{15}N enrichment*
1. Resting cells	100%	0.10
2. Broken cells not centrifuged	8 %	0.64
3. Extract 27,000 × *g*/15 min	None	0.54
4. Extract 144,000 × *g*/90 min	None	0.78

Air sensitivity is marked, although serious losses in activity were not encountered during brief exposures to air provided the enzyme solution was kept cold and not agitated during the exposure. Particular care must be taken to exclude O_2 during the fixation assay procedure. For example, a significant drop in fixation occurred from air released from small ice particles not melted prior to sealing the preparation with $^{15}N_2$.

Ionic strength also affects activity. Good fixation can be achieved in the 0.05 *M* phosphate buffer (pH 6.8), but this concentration apparently represents a compromise

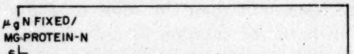

Fig. 2. Effect of pH on nitrogen-fixing activity. The reaction mixture contained 12 mg protein-N as cell-free extract, 100 mg sodium pyruvate, and 0.05 *M* phosphate buffer at the desired pH in total volume of 8 ml. The solutions were incubated under 0.5 atm of 60 atom% $^{15}N_2$ 60 min at 30°. Extracts were prepared in the buffer at pH 6.8 as usual and adjusted to desired pH just before use.

between the requirements for pH control and sensitivity to ionic strength. Higher concentrations of phosphate were deleterious. Addition of sodium chloride to the buffer to bring it to 0.05 M NaCl and 0.05 M phosphate similarly caused substantial losses of activity.

A point of significance relative to the mechanism of fixation is that the enzyme system apparently does not effect the isotope exchange reaction of N_2. Experiments (Table III), which should be repeated with refinements, have thus far failed to provide evidence that this reaction occurs under influence of the extracts either in the presence or absence of added sodium pyruvate. Accordingly, dissociation of N_2 may not be an early step in adsorption.

TABLE III

ISOTOPE EXCHANGE EXPERIMENTS $^{14}N_2 + {}^{15}N_2 \leftrightharpoons 2{}^{14}N—{}^{15}N$

Conditions	*% $^{14}N_2$*	*% ^{14}N–^{15}N*	*% $^{15}N_2$*
1. Control sample before exposure to cell-free preparation	94.10	3.57	2.39
2. Control sample exposed 1 h to cell-free preparation with pyruvate added*	94.00	3.63	2.41
3. As above without pyruvate	94.00	3.69	2.39
4. Calculated equilibrium composition	91.86	7.97	0.17

* This preparation fixed 0.14 atom % excess ^{15}N when exposed to 60 atom % excess $^{15}N_2$ under the condition of 2.

Dependence of fixation activity on pyruvate metabolism

Nitrogen fixation in the extracts is closely coupled with pyruvate metabolism though the mode of coupling has yet to be learned. A search for other compounds capable of supporting nitrogen fixation turned up only one, α-ketobutyrate, but this compound is only about half as effective on a molar basis as pyruvate. The majority of compounds tested (Table IV) were selected on the possibility that they might

TABLE IV

COMPOUNDS TESTED FOR PYRUVATE REPLACEMENT

Compound added	*Atom % excess ^{15}N*
None	0.00
Pyruvate (100 mg)	0.64
α-ketobutyrate (100 mg)	0.25
Hydrogen (0.7 atm)	0.00
Hydrogen (0.7 atm) + pyruvate (100 mg)	0.60

* Flasks contained in addition to the compounds mentioned, the cell-free extract (15 mg protein-N), 0.3 atm of 60 atom % $^{15}N_2$ and buffer to make 8 ml. Time of incubation was 1 h.

Other compounds tried which would not support nitrogen fixation were sodium or potassium salts of formic, acetic, propionic, butyric, glycolic, glyoxylic, lactic, crotonic, malic, oxalacetic, succinic, fumaric, mesaconic, α-ketoglutaric, phosphopyruvic, and phenylpyruvic acids, ethanol, propanol, glycerol, 2,3-butanediol, acetoin, biacetyl, acetaldehyde, ornithine, sucrose, ethyl acetoacetate, and the substrate-enzyme combinations, malic acid/malic acid dehydrogenase and glucose-6-phosphate/glucose-6-phosphate dehydrogenase.

serve as hydrogen donors, ammonia acceptors or pyruvate precursors. Also tested were various products of pyruvate metabolism, a few of which proved inhibitory to fixation (Table V). Presumably the function of pyruvate is to supply reducing capacity to the fixation system. A detailed understanding of pyruvate chemistry in the extracts should assist in unraveling the mechanism of coupling to fixation.

TABLE V

EFFECTS OF METABOLITES ON NITROGEN FIXATION IN EXTRACTS

*Compound added**		*Nitrogen fixation as % of control*
Sodium butyrate	(100 mg)	71
Sodium acetate	(100 mg)	75
Lithium acetyl phosphate	(12 mg)	50
Lithium acetyl phosphate	(25 mg)	0
ATP	(4 mg)	80
ATP	(20 mg)	0
Phosphate	(300 µmoles)	100
Phosphate	(400 µmoles)	100
Phosphate	(900 µmoles)	45
Phosphate	(1400 µmoles)	15

* Each flask also contained cell-free extract (15–20 mg protein-N), 100 mg sodium pyruvate 0.3 atm of 60 atom % $^{15}N_2$, and total volume was adjusted to 8 ml with buffer. Time of incubation was 1 h. Control fixed 0.51 atom % excess ^{15}N.

There is an optimum concentration of pyruvate for maximum fixation activity, and the amount varies with the concentration of protein in the extracts. Normally, the fixation assay system contained 2 mg protein-N/ml, and maximum fixation was attained with sodium pyruvate at 12 mg/ml. For more dilute extracts, the optimum pyruvate concentration was lower. These relationships are illustrated in Tables VI and VII and in Fig. 3.

Time-course experiments revealed that the start of nitrogen fixation lagged behind the start of pyruvate metabolism, which began with a burst several times

TABLE VI

EFFECTS OF PYRUVATE CONCENTRATION AND CENTRIFUGATION ON NITROGEN FIXATION IN EXTRACTS

*Flask**	*Pyruvate (mg)*	*Preparation***	*^{15}N fixed (µg)*
1	200	Uncentrifuged	50
2	100	Uncentrifuged	48
3	50	Uncentrifuged	32
4	200	Centrifuged	33
5	100	Centrifuged	84
6	50	Centrifuged	46

* Each flask contained also 0.6 atm of 60 atom % $^{15}N_2$ and enough buffer to give 8 ml total volume. Time of incubation was 1 h. Equivalent volumes of uncentrifuged to centrifuged preparation were used; 20.2 mg protein-N/flask for the uncentrifuged preparation and 14.5 mg protein-N/flask for the centrifuged preparation.

** The uncentrifuged preparation metabolized pyruvate at a rate 150 % that of the centrifuged preparation as measured by H_2 evolution.

faster than the rate it maintained after fixation got underway. Data are presented in Fig. 4. The rates of nitrogen fixation and pyruvate metabolism were linear after the induction period. The significance of the initial lag in fixation and of the initial high rate of pyruvate consumption has not been established. The induction period may represent the time required to generate a key substrate or to condition in some way an enzyme essential to the nitrogen fixation system. Spectral responses discussed below point to the latter possibility.

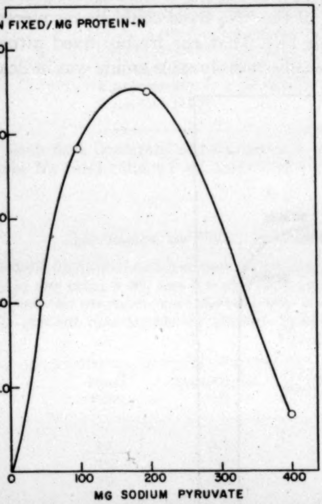

Fig. 3. Effect of concentration of pyruvate on nitrogen-fixing activity. The system was identical to that in Fig. 2 except 20 mg protein-N as cell-free extract was used in each determination and the solution was buffered at pH 6.5.

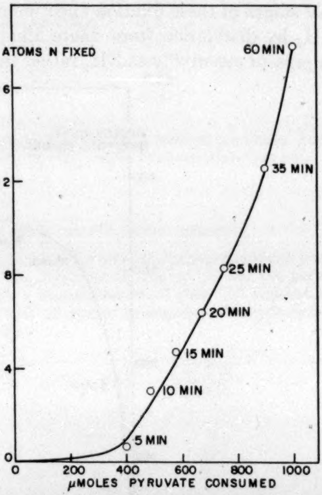

Fig. 4. Time-course of nitrogen fixation and pyruvate consumption. The reaction mixture of 8 ml total volume contained 14 mg protein-N as cell-free extract and 100 mg sodium pyruvate in 0.05 *M* phosphate buffer pH 6.5. The mixture was incubated with 0.5 atm of 60 atom % $^{15}N_2$ for the varying periods indicated and then analyzed for ^{15}N and pyruvate[36].

That it was the metabolism of pyruvate rather than the presence of pyruvate *per se* that gave rise to nitrogen fixation in the extracts seems fairly well indicated by the time-course data and by the fact that α-ketobutyrate also was active.

Chemistry of pyruvate in the extracts

In view of the requirement for pyruvate and the nature of some of the products formed, it would seem that nitrogen fixation may be coupled to the phosphoroclastic reaction. The phosphoroclastic reaction, which occurs in various clostridia[30, 31], appears to operate in the nitrogen-fixing extracts. H_2 and CO_2 are copiously evolved in 1:1 ratio when the extracts are supplied with pyruvate. Formation of acetyl phosphate in substantial quantities has also been observed by the hydroxamic acid method[32] (Fig. 5). Acetyl phosphate is an inhibitor for nitrogen fixation (Table V), but its effect is limited by reactions converting it to other products and preventing its accumulation. Coenzyme A, required by the phosphoroclastic system, was frequently stimulatory to nitrogen fixation by these extracts (Table VIII). Other supplements implicated in pyruvate metabolism such as iron, FAD, FMN, cocarboxylase, DPN, TPN, lipoic acid, and folic acid were not effective in the crude extracts.

Chemistry of $^{15}N_2$ in the nitrogen-fixing extracts

In the *C. pasteurianum* extracts the product of nitrogen fixation is NH_3. During early stages of these fixation experiments, all of the $^{15}N_2$ fixed could be recovered as $^{15}NH_3$ by distillation from dilute alkali (Table IX). That the freshly fixed nitrogen was present essentially as NH_3 rather than as easily hydrolyzable amide was indicated

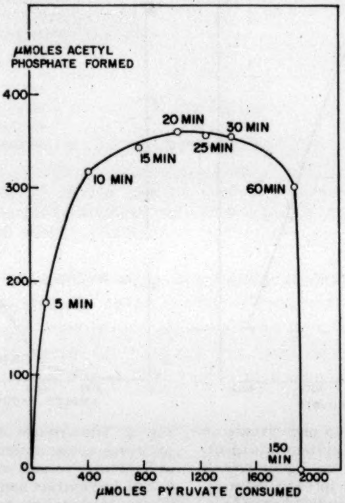

Fig. 5. Time-course of pyruvate consumption and acetyl phosphate content. Procedure was similar to that in Fig. 4. Fixation was interrupted after the varying periods indicated, and the solutions were analyzed for pyruvate[36] and acetyl phosphate[32].

TABLE VII

EFFECT OF PROTEIN CONCENTRATION ON NITROGEN FIXATION IN *C. pasteurianum* EXTRACTS

Pyruvate (mg)	*Protein-N* (mg)*	*Atom % excess ^{15}N*
100	21.7	0.48
100	15.5	1.05
100	9.3	1.39
100	3.1	0.02

* Extract was centrifuged at 144,000 × *g* and aliquots were taken to give protein concentrations indicated. Each flask was made to 8 ml with buffer, and 0.6 atm of 60 atom % $^{15}N_2$ was used. Time of incubation was 1 h.

TABLE VIII

EFFECT OF SUPPLEMENTS IN *C. pasteurianum* EXTRACTS

*Additions to standard assay system**	*Atom % excess ^{15}N*
None	0.53
10 μmoles CoA	0.65
5 μmoles Fe^{++}	0.51
Cocarboxylase (1 μmole)	0.53
Riboflavin-5-PO_4 (10 μmoles)	0.53
Cocarboxylase, CoA, FMN, Fe^{++}	0.61
CoA, (5 μmoles), 2 μmoles Fe^{++}, 2 μmoles FMN	0.63
CoA (5 μmoles), 2 μmoles FMN	0.63
2 μmoles Mo as $Na_2MoO_4 \cdot 2H_2O$	0.56
2 μmoles Mo + 2 μmoles Fe^{++}	0.53
2 μmoles DPN	0.52

* Each flask contained cell-free extract (15 mg protein-N), 100 mg sodium pyruvate, buffer to make the total volume 8 ml, and 0.6 atm of 60 atom % $^{15}N_2$. Time of incubation was 1 h.

TABLE IX

CONVERSION OF $^{15}NH_3$ TO DERIVATIVES WITH TIME DURING FIXATION

Each flask contained cell-free extract (15 mg protein-N), 100 mg sodium pyruvate, enough buffer to make the total 8 ml, and 0.6 atm of 60 atom % $^{15}N_2$. After incubation at 30° for the periods indicated, the solutions were divided into two parts for determination of total ^{15}N content and of ^{15}N content recoverable by alkaline distillation. The difference represented nonhydrolyzable ^{15}N derivatives.

Time (min)	*Total ^{15}N fixed (μg)*	*^{15}N recovered by alkaline distillation (μg)*	*% ^{15}N as $^{15}NH_3$*	*% ^{15}N not hydrolyzable to $^{15}NH_3$*
12	109	109	100	0
20	190	85	45	55
30	244	10	4	96

TABLE X

DISTRIBUTION OF ^{15}N AMONG NH_3, AMIDE AND OTHER FORMS IN NITROGEN-FIXING EXTRACTS

Fixation mixture comprised cell-free extract (16.8 mg protein-N), 100 mg sodium pyruvate, and buffer to make 8 ml.

Forms of nitrogen	*Amounts of nitrogen*		
	Before fixation (μg)	*After fixation (μg)*	*Gain (μg)*
As NH_3*	22.4	258	236
As amide*	437	442	5
Unavailable by alkaline distillation	—	—	6
Total fixed**	—	247	247

* Ammonia and ammonia-plus-amide contents before and after fixation were determined on separate aliquots by distillation from K_2CO_3 and from NaOH, respectively, in micro-Conway dishes[33]. The difference represented amide content.

** Total nitrogen gain of 247 μg was determined from ^{15}N enrichment of 0.94 % resulting from exposure for 1 h to 60 atom % excess $^{15}N_2$. Difference between 247 and sum of ammonia and amide nitrogen contents (241) gave amount of fixed nitrogen converted to forms not recoverable as NH_3 by alkaline distillation.

by tests summarized in Table X. Of the total nitrogen fixed, 95% was present as ammonia and about 2% as amide, with the remainder in non-hydrolyzable though Kjeldahl-digestible forms.

Whether there are intermediates between N_2 and NH_3 remains undecided. Certainly, unless a 6-electron transfer occurs, partially reduced forms of N_2 must intervene, although they may be retained on the enzyme until converted to NH_3. Attempts to detect possible fixation intermediates such as hydrazine, hydroxylamine, and nitrite have been unsuccessful. The data on $^{15}NH_3$ recovery argue against a carbon derivative such as dihydropyridazinone-5-carboxylic acid[11] as an intermediate of nitrogen fixation. If it were involved, only 50% of the ^{15}N fixed should be recoverable as free NH_3 by simple hydrolysis. Furthermore, tests indicated that this compound was not metabolized by the extracts.

TABLE XI

RETENTION OF ^{15}N AMMONIA BY TRAPPING AGENTS IN NITROGEN-FIXING EXTRACTS

Expt.	*Additions to standard assay system*	*^{15}N fixed*	
		Total (μg)	*Recovered by alkaline distillation (μg)*
1a	None	244	10
1b	1.7 mg N as NH_3	214	182
1c	4.0 mg N as amide in glutamine	240	250
2a	None	157	2
2b	4 mg N as NH_3	78	51
2c	4 mg N as amide in glutamine	138	118
3a	None	173	163
3b	4 mg N as NH_3	55	54
3c	100 mg α-ketoglutarate	66	2
3d	100 mg α-ketoglutarate + 4 mg N as NH_3	14	12

Procedures were as for Table IX. Time of incubation 1 h.

Cell-free fixation experiments allowed to proceed more than 10–15 min often contained most of the ^{15}N in a form no longer released by alkaline distillation (Table IX). This effect did not always occur, as apparently the extracts varied in their ability to incorporate NH_3 into organic compounds. Conversion of $^{15}NH_3$ into organic derivatives could be largely suppressed by supplying an initial pool of NH_3 or glutamine to trap by dilution the freshly fixed nitrogen (Table XI). Glutamine had little or no effect on nitrogen fixation, but NH_3 was inhibitory at high concentrations (Table XII). From this, the NH_3 acceptor mechanism could presumably have an important role in determining fixation yield. However, the minimum inhibitory concentration of NH_3 exceeded the levels normally produced in the extracts, and it would therefore appear that NH_3 acceptors were not a rate-limiting factor in nitrogen fixation under present conditions. Specifically, inhibition by NH_3 became detectable when the concentration reached about 200 μg ammonia-N/ml whereas the normal yield was about 30 μg fixed-N/ml.

Extracts varied considerably in ability to convert freshly fixed nitrogen to nonhydrolyzable derivatives; an example is seen in Table XI, 3a, wherein over 90%

of the fixed nitrogen remained as NH_3 or amide after 60 min. Conversion of freshly fixed nitrogen to nonhydrolyzable derivatives was accelerated by addition of α-ketoglutarate, a known NH_3 acceptor (Table XI, Expt. 3); at the same time, nitrogen fixation was strongly inhibited. An interference with the reduction system is assumed to have occurred, perhaps by antagomism of pyruvate or interception of hydrogen, but the mechanism is not known.

TABLE XII

AMMONIA-INHIBITION OF NITROGEN-FIXATION IN EXTRACTS

Ammonia-N added/flask *	*% Inhibition*
1.7 mg	10–20 %
4.0 mg	50–60 %
20.0 mg	94–98 %

* Mixture consisted of cell-free extract (15–20 mg protein-N), 100 mg sodium pyruvate, buffer to make 8 ml, and 0.5 atm of 60 atom $^{15}N_2$. Inhibition was determined by comparison of ^{15}N fixed with controls.

To provide an indication of the types of organic nitrogen compounds formed in the crude extracts, the distribution of ^{15}N among various free amino acids was determined[27] by fractionation of the deproteinized extract. The highest concentration of label was in glutamic acid, 21.7 atom % excess ^{15}N, indicating that about one-third of the glutamic acid present was formed from freshly fixed nitrogen. The next most heavily labeled amino acid was not identified but contained only 5.56 atom % excess ^{15}N.

Spectral effects of nitrogen in the extracts

SHUG, HAMILTON AND WILSON[4] have shown that sonic extracts of *C. pasteurianum* undergo spectral changes at 390–450 mμ on exposure to N_2. These authors[34] and later BERGERSEN AND WILSON[35] observed similar effects in sonic extracts of *Azotobacter*

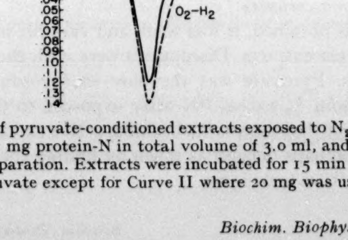

Fig. 6. Difference spectra of pyruvate-conditioned extracts exposed to N_2, A, and O_2 relative to H_2. The extracts contained 1.1 mg protein-N in total volume of 3.0 ml, and their spectra were determined within 2 h after preparation. Extracts were incubated for 15 min at 25° with 40 mg sodium pyruvate except for Curve II where 20 mg was used.

vinelandii and soybean root nodules, in which N_2-induced spectral changes were found at 400 and 550 mμ.

N_2 has now been found to cause characteristic spectral changes in the nitrogen-fixing enzyme preparations from *C. pasteurianum*. Freshly prepared extracts conditioned with sodium pyruvate (40 mg/ml) or sodium α-ketobutyrate exhibit an increase in absorption at 365 mμ when exposed to N_2. The effect is illustrated in Fig. 6, which gives the difference spectra of such preparations measured under N_2, A, and O_2 relative to H_2. Responses to A and O_2 (decreased absorption at 365 mμ) were also obtained and may have been caused by desorption of H_2 since they were reversed by replacing A or O_2 with H_2 in the overlying atmosphere (Fig. 7). The N_2 response was not similarly reversible. In the absence of pyruvate or α-ketobutyrate, the N_2-induced spectral change was located at about 300 mμ rather than at 365 mμ and has been rather erratic in occurrence.

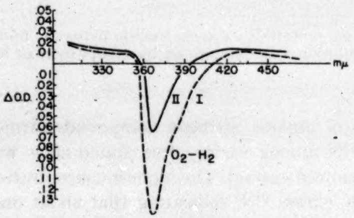

Fig. 7. Difference spectra of pyruvate-conditioned extracts exposed to O_2 reversed by H_2. Curve I represents the difference spectrum after 15 min exposure to O_2 at 0°. Curve II represents the difference spectrum as in I followed by exposure to H_2 for 10 min.

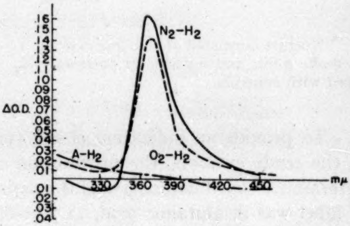

Fig. 8. Difference spectra of pyruvate-conditioned extracts under N_2, A, and O_2 relative to controls under H_2. Extracts were stored 16 h under H_2 before use.

In extracts stored more than 7 h at 5° under H_2, the N_2-induced spectral change decreased with time until after 36 to 48 h it was no longer detectable. Also, the apparent reversible adsorption of H_2 disappeared and the O_2 response became indistinguishable from that of N_2 (Fig. 8). Nitrogen-fixing activity of the extracts decreased to zero at approximately the same time.

These spectral changes are believed to be evidence for the interaction of N_2 with the nitrogen-fixing enzyme system. Their significance to the mechanism of nitrogen fixation is being investigated.

Trial extraction of other organisms

A few attempts were made to obtain nitrogen fixation in extracts of *Azotobacter vinelandii* and *Nostoc muscorum* G.

While activity was obtained, it was weak and variable in contrast to the results with the *C. pasteurianum* extracts. Disruptions were with the Hughes press and also with sonic oscillations. Pyruvate was the only energy source tried. Enrichments were less than 0.06 atom % excess ^{15}N after exposure to 60 atom % excess $^{15}N_2$ for 1 h. One might expect that success in obtaining active extracts from other nitrogen-fixing organisms will perhaps depend as much on finding the proper activator as on using the proper extraction techniques.

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REFERENCES

1 R. H. Burris, F. J. Eppling, H. B. Wahlin and P. W. Wilson, *J. Biol. Chem.*, 148 (1943) 349.
2 P. G. Hamilton, W. E. Magee and L. E. Mortenson, *Bacteriol. Proc.*, 82 (1953)
3 R. H. Burris, in W. D. McElroy and B. H. Glass, *Symposium on Inorganic Nitrogen Metabolism*, Johns Hopkins Press, Baltimore, 1956, p. 316.
4 A. L. Shug, P. B. Hamilton and P. W. Wilson, in W. D. McElroy and B. H. Glass, *Symposium on Inorganic Nitrogen Metabolism*, Johns Hopkins Press, Baltimore, 1956, p. 344.
5 G. E. Hoch and D. W. S. Westlake, *Federation Proc.*, 17 (1958) 243.
6 A. Nason and H. Takahashi, *Ann. Rev., Microbiol.*, 12 (1958) 203.
7 I. Zelitch, E. D. Rosenblum, R. H. Burris and P. W. Wilson, *J. Biol. Chem.*, 191 (1951) 295.
8 J. W. Newton, P. W. Wilson and R. H. Burris, *J. Biol. Chem.*, 204 (1953) 445.
9 P. W. Wilson and R. H. Burris, *Ann. Rev. Microbiol.*, 7 (1953) 415.
10 A. I. Virtanen, *Angew. Chem.*, 65 (1953) 1.
11 M. K. Bach, *Biochim. Biophys. Acta*, 26 (1957) 104.
12 E.R. Roberts, *Symposium of the Society for Experimental Biology*, Vol. 13, Academic Press, New York, 1959, p. 24.
13 F. E. Mumford, J. E. Carnahan and J. E. Castle, *J. Bacteriol.*, 77 (1959) 86.
14 P. W. Wilson, in C. H. Werkman and P. W. Wilson, *Bacterial Physiology*, Academic Press, New York, 1951, Ch. 14.
15 E. D. Rosenblum and P. W. Wilson, *J. Bacteriol.*, 59 (1950) 83.
16 D. C. Pratt and A. W. Frenkel, *Plant Physiol.*, 34 (1959) 333.
17 H. L. Jensen and D. Spencer, *Linnean Society N.S. Wales*, 72, pts. 1–2 (1947) 73.
18 R. G. Esposito and P. W. Wilson, *Proc. Soc. Exptl. Biol. Med.*, 93 (1956) 564.
19 J. E. Carnahan and J. E. Castle, *J. Bacteriol.*, 75 (1958) 121.
20 J. E. Carnahan, L. E. Mortenson, H. F. Mower and J. E. Castle, *Biochim. Biophys. Acta*, 38 (1960) 188.
21 K. C. Schneider, C. Bradbeer, R. N. Singh, Li Chuan Wang, P. W. Wilson and R. H. Burris, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 726.
22 D. E. Hughes, *Brit. J. Exptl. Pathol.*, 32 (1951) 97.
23 R. H. Burris and P. W. Wilson, *Methods in Enzymology*, Vol. 4, Academic Press, Inc., New York, 1957, p. 355.
24 A. Steyermark, *Quantitative Organic Microanalysis*, Blakiston Publishing Co., Inc., New York, 1951, p. 152.
25 G. W. Watt and J. D. Chrisp, *Anal. Chem.*, 24 (1952) 2006.
26 G. Endres and L. Kaufmann, *Ann. Chem., Liebigs*, 530 (1937) 184.
27 J. S. Wall, *Anal. Chem.*, 25 (1953) 950.
28 R. J. Block, E. L. Durrum and G. Zweig, *A Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press, Inc., New York, 1955, p. 75.
29 C. Lamanna and M. F. Mallette, *J. Bacteriol.*, 67 (1954) 503.
30 R. S. Wolfe and D. J. O'Kane, *J. Biol. Chem.*, 205 (1953) 755.
31 A. L. Shug and P. W. Wilson, *Federation Proc.*, 15 (1956) 355.
32 F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, 159 (1945) 21.
33 E. J. Conway, *Microdiffusion Analysis and Volumetric Error*, Crosby Lockwood and Son, Ltd., London, 1947.
34 P. B. Hamilton, A. L. Shug and P. W. Wilson, *Proc. Natl. Acad. Sci. U.S.*, 43 (1957) 304.
35 F. J. Bergersen and P. W. Wilson, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 1641.
36 T. E. Friedman and G. E. Haugen, *J. Biol. Chem.*, 147 (1943) 415.

THE LOCALIZATION OF INTRACELLULAR ORTHOPHOSPHATE: THE ROLE OF THE NUCLEOLI

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SUMMARY

Intracellular orthophosphate has been identified as the fraction which precipitates in the nucleoli after Pb acetate fixation. The results are in good agreement with the important role played by the nucleus—and specially the nucleolus—in the phosphorus metabolism of the cell. On the other hand it is not known how much of the total orthophosphate of the whole cell is present in the nucleoli.

INTRODUCTION

Inorganic phosphate has been localized in the nucleolus by a cytochemical method[1-3]. The unexpected result occurs that when lead acetate is employed as a fixing fluid, a precipitate of lead phosphate appears in the nucleoli. It is clear that lead acetate is able to precipitate the phosphate ions inside the cells; this is, to our knowledge, the first case in which a small, diffusible ion has been localized at the cellular level. The following work was carried out in order to examine this phenomenon more closely.

MATERIALS AND METHODS

The materials used consisted of onion, maize and broad bean roots, ungerminated broad bean embryos, the large internodal cells of *Nitella clavata* and the livers of guinea pigs and chickens. The cytochemical method used was essentially the same as previously described[1]. Tissue extracts were prepared with ice cold 5 % TCA, 4 % perchloric acid or 50 % ethanol plus 5 % acetic acid for 30 min. 5 to 10 ml of extracting fluid were used per g of wet weight tissue. The tissues were not homogenized so that the results may be compared more directly with the cytochemical data. Acetic acid–ethanol and TCA extracts (TCA removed with ethyl ether) were employed either immediately or concentrated over $CaCl_2$ for 24 h at room temperature. An ascending chromatogram was obtained with Whatman No. 1 paper washed with 10 % acetic acid and glass bi-distilled water[4] and a solvent consisting of acetone–acetic acid–water (50:15:35)[5,6]. This solvent, reported for the chromatography of phosphorylated compounds, was found to give good separation with phenolic and ninhydrin-positive substances also. The spots were detected by the application of several color reagents using the "dipping" and "multiple dipping" procedures[7,8]. Pb-precipitating substances were detected by drawing the chromatogram through a 5 % sol. and allowing it to hang for 15 min. Three washings with bidistilled water, 5 to 10 min each time, were found necessary in order to remove the excess of Pb from the paper; after evaporation of the water the chromatogram was suspended in an H_2S atmosphere. Pb-precipitated substances appeared immediately as brown spots. Pb orthophosphate can be shown on the same paper by dipping the paper into the molybdate reagent[5]: the PbS spot is transformed after a few minutes into a blue spot of phosphomolybdate. Pb precipitates, insoluble in acetic acid, were detected by immersing the Pb acetate dipped-chromatogram into a 5 % Pb acetate solution in glacial acetic acid (*cf.*[1]) for 1 h and proceeding as before. Azide-iodine[9] acts as an iodine stain[10]; brown spots were detected in those cases where no color was seen with iodine alone. Ultraviolet absorbing spots were detected by GORDON's blueprint paper method[11]. Orthophosphate and the total acid-soluble phosphates were measured by the method of KING[12]. Ribonuclease (Sigma, crystalline) was employed at a concentration of 5 mg/ml distilled water.

RESULTS

The chemical nature of the nucleolar precipitate

Table I shows that very probably the Pb-precipitable substances are extracted by cold TCA. After Pb acetate treatment of TCA fixed roots, a PbS stain is developed in the whole root (approximately 1 mm thick) but no precipitate is found in the nucleoli. If treatment with Pb in glacial acetic acid is made after the aqueous Pb acetate the roots remain completely unstained. This shows (a) that all the acid-insoluble compounds which show affinity for Pb are extracted by the Pb-acetic acid and (b) that very probably the nucleolar substance does not remain in the tissue in a diffused condition, *i.e.*, it must be present in the TCA extract. Immersion in liquid nitrogen is not as effective as Pb fixation: after Pb acetate treatment the phosphate is preserved in a diffused condition in the tissue. The freezing-substitution procedure of RUSSELL *et al.*[13] in which basic Pb acetate is added to the chilled ethanol, is also

TABLE I

REACTIVITY OF LEAD WITH ACID-SOLUBLE AND ACID-INSOLUBLE FRACTIONS IN ONION AND MAIZE ROOTS AFTER SEVERAL TREATMENTS

Fixation	*Post-treatment*	*Precipitate in the nucleoli*	*PbS stain in the whole root*
Pb acetate		+	+
Pb acetate	Pb in acetic acid	+	+
Cold TCA	Pb acetate	—	+
Cold TCA	Pb acetate, then Pb in acetic acid	—	—
Pb acetate	Cold TCA, then Pb acetate, then Pb in acetic acid	—	—
Liquid N_2	Pb acetate, then Pb in acetic acid	— (some + diffusion figures)	+

(+) or (—) indicate whether the Pb-precipitate is present or absent from the nucleoli and, in the case of the PbS stain, whether the roots are stained black or remain unstained.

TABL

COLOR REACTIONS APPLIED TO THE CHROMATOGRA

Spots numbered accordin

Spot No.	*Pb ac.*	*Pb ac.-acetic acid*	*Uranyl ac.*	*Molybdate*	*u.v. absorbing*	*$FeCl_3$*	*Diazotized sulfanilic acid*	*Diazotized p-nitroaniline*	*Gibbs*
10	+				+	violet	red-orange	violet	blue
9	+				+	violet	red-brown	violet	yellow
8									
7, 7′					+?	yellow	light red	yellow	yellow
6									
5	+	+	+	+					
4									
3							pink	violet	
2, 2′							pink	violet	
1							pink	violet	

ineffective in precipitating the phosphate at the cellular level. Fig. 1 shows a diagram of a one-dimensional ascendent chromatogram of acid-soluble substances in maize roots (var. Colorado Klein). At least 12 substances can be detected by the application of several color reactions, but only 3 give a visible precipitate with Pb acetate; of these only one, orthophosphate, is insoluble in Pb-acetic acid. The same can be said in the cases of onion and broad bean roots. Spots No. 9 and 10, which form Pb precipitates soluble in acetic acid, are flavonoid compounds[14] for they give typical reactions for phenolic substances and a positive reaction for ketones with 2,4-dinitrophenyl hydrazine. *o*-diphenols are known to precipitate with neutral Pb acetate and to

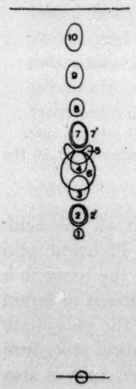

Fig. 1. Pattern of acid-soluble substances in maize roots (cold TCA or acetic-ethanol extracts). Whatman No. 1, ascending for 24 h; solvent, acetone-acetic acid-water (50:15:35); see Table II for detection of the spots; only spots No. 5 (orthophosphate), 9 and 10 (flavonoid compounds) are present in appreciable enough amounts to give a precipitate with Pb acetate; of these, only orthophosphate forms a Pb precipitate insoluble in a 5% Pb acetate solution in glacial acetic acid.

II

OF ACID-SOLUBLE SUBSTANCES IN MAIZE ROOTS

to diagram in Fig. 1.

α-nitroso β-naphtol	*Iodine*	*Azide-iodine*	*Alkaline $AgNO_3$*	*2,4-dinitro phenyl hydrazine*	*Ninhydrin*	*Isatin*	*2,6-dichloro phenol-indophenol*	*Bromocresol green*	*Basic Pb ac.*
red	+	+	brown	+			purple (bleaches)		light yellow
yellow	+	+	brown	+			purple (bleaches)		light yellow
		+?			blue				
red	+	+		+?	yellow	blue	blue	blue	
			black						
		+			blue				
		+			blue		purple		
		+			yellow		purple		
		+			blue				

strongly reduce the alkaline silver reagent[15]. This is not the present case since only a brown spot is obtained with the silver reagent; the precipitating property is rather due to the relative positions of the OH-groups and CO-groups[16]. They are easily detected as strongly u.v. absorbing spots or as violet and light yellow spots with $FeCl_3$ and basic lead acetate[17] respectively; they give different colors with diazotized sulfanilic acid. On the basis of the typical blue color given by Gibbs reagent (2,6-dibromoquinonechloroimide) and the red spot obtained with α-nitroso-β-naphthol it is concluded that only spot No. 10 has unsubstituted *para* and *ortho* positions. In onion and broad bean roots this fraction is found in a position just above the orthophosphate spot; they also give a Pb precipitate soluble in acetic acid and a positive ketone reaction, but a blue color with Gibbs reagent and a violet spot with $FeCl_3$ is given by the broad bean only. Substances with these characteristics have not been found in liver extracts. The same chromatographic pattern is obtained when the tissues are submitted to extraction with ethanol–acetic acid or when Pb acetate-fixed roots are extracted with cold TCA; in the last case a drop of a concentrated solution of ethylenediamine (EDTA) is added to prevent precipitation of lead as the TCA is gradually removed with ethyl ether. Spots Nos. 1, 2, 3, 4, 7 and 8 are ninhydrin-reacting substances; spots Nos. 2 and 7 give a yellow color instead of the blue color characteristic of the great majority of amino acids. A parallel run with asparagine showed that it has the same R_F as spot No. 2 and that spot No. 7 is an isatin-reacting substance. On this basis, and because they have been reported in the maize anther[18], spots Nos. 2 and 7 were identified as asparagine and proline respectively. Neither of these ninhydrin-reacting substances is tryptophan or arginine since no reaction can be detected on the paper chromatogram with the Ehrlich or Sakaguchi reagents. Asparagine was also detected in broad bean and onion roots. Another substance with the same R_F as asparagine is present in position 2 (spot No. 2′); it gives an intense pink color with diazotized sulfanilic acid and a violet spot with diazotized nitroaniline. Another substance is also present in position 7 (spot No. 7′); it is a basic compound for it gives blue spots with the pH indicator reagents. That we have here two substances is also indicated by the fact that in onion and broad bean roots, as well as in the liver, spot No. 7 (proline) is absent while the basic compound (spot No. 7′) is present. The large spot No. 6 is due to sugars. Phosphate analysis on cold 5 % TCA and 4 % perchloric acid of maize and onion roots showed that orthophosphate accounts for 60–65 % of the total acid-soluble phosphates; there is almost twice as much orthophosphate per g of wet weight tissue in onion as in maize roots. Organic phosphates are present in amounts too small for detection on the paper chromatogram by chemical means (*cf.*[19]). Similar high values for orthophosphate have been found in barley roots and seedlings[20, 21]. It is concluded that orthophosphate is the only substance present in the tissue in appreciable enough amounts to give a visible precipitate at the cellular level (Pb salt insoluble in Pb-acetic acid). In the liver, where large quantities of extracellular phosphate are present, the cytological image shows a granular precipitate in the intercellular spaces and specially around the central veins. With perfused liver practically all the "granular" precipitate is absent from the tissue while the dense nucleolar precipitate persists (extracellular phosphate washed out by perfusion with physiological saline). It is concluded that the fraction precipitated by Pb acetate within the nuclei is intracellular orthophosphate.

The nucleolar precipitate in modified cells

Pb acetate fixation of ribonuclease-treated (*in vivo*, 8 h) onion roots shows that phosphate precipitates in the nucleoli even when nucleolar basophilia is lost (staining for RNA with toluidine blue after Carnoy fixation). This is in agreement with the finding that ribonuclease neither markedly affects the inorganic phosphate content[22] nor the uptake of ^{32}P in onion roots[23]. In the large intact cell of *Nitella* the lead precipitate appears specifically located in the nucleoli; more than 100 nuclei can be seen in one large cell and each nucleus contains approximately 20 to 50 Pb-positive nucleoli (Fig. 2). There is no indication of Pb-precipitates in the nuclear membrane, cytoplasm or in the large central vacuole; during nuclear division by amitosis[24] the nucleoli remain Pb-positive. In cut cells most of the vacuolar sap flows out but the protoplasm remains within the cell wall; the results are usually the same as in the intact cell. Since the integrity of the cell membrane is destroyed this suggests that the membrane is not significantly involved in the retention of orthophosphate and that it is "bound" intracellularly. Moreover, the membrane cannot act as a barrier to lead penetration; it is interesting that, according to OSTERHOUT[25, 26] 0.01 *M* Pb acetate is not toxic to *Nitella* (it stops protoplasmic motion and contracts the chloroplasts but these effects can be reversed by washing with distilled water). Although the cell's metabolic activity is a complicating factor, it is possible that Pb acetate applied at a sufficiently high concentration enters the cell by physical processes only; this certainly applies to physiologically dormant cells. Excised ungerminated broad bean embryos show the characteristic Pb phosphate precipitate in the nucleoli after Pb fixation (either ice cold or at room temperature). On the other hand, no precipitate is formed in dividing cells at those stages of mitosis where the nucleolus is absent; this result has a bearing on the strongly decreased uptake of ^{32}P in dividing cells[27].

Fig. 2. A nucleus of the multinucleate internodal cell of the fresh-water alga *Nitella clavata* showing the nucleolar localization of orthophosphate; there are more nucleoli in other planes which are not seen in the photograph. Cut cells show the same pattern of localization as in the intact living cell. Fixation in 5% Pb acetate, followed by treatment with 5% Pb acetate in glacial acetic acid, washing in distilled water and staining with H_2S; total mount. The photograph represents magnification 2430 ×.

DISCUSSION

The ability to precipitate orthophosphate inside the cell is a characteristic of the nucleolus itself, since nothing is precipitated at meta- and anaphase when this structure is absent. Since the phosphate precipitate is found specifically in the nucleoli it is difficult to avoid the conclusion—and it is the simplest explanation—that orthophosphate is present in the nucleolus. Of course, a negative result does not mean that inorganic phosphate is absent from the cytoplasm since the degree of dispersion can affect its visualization very markedly. This means that if the number of orthophosphate ions per unit volume (concentration) is greater in the nucleolus than in the cytoplasm, "cytochemically detectable amounts" will be found only in the nucleolus even though it might be only a small fraction of the total orthophosphate of the whole cell. A large number of investigations have established that the nucleus—and specially the nucleolus—plays an important role in the phosphorus metabolism of the cell; it is very likely that the presence of orthophosphate in appreciable amounts in the nucleolus (nuclear orthophosphate) is connected with: (a) high metabolic and specific activities of the nucleolar RNA[28–35], (b) the dependence of the nucleus on the net synthesis of cytoplasmic RNA[36–38], (c) the synthesis of other nucleotides besides RNA[39, 40], (d) the specificity of localization of DNA in the nucleus and (e) the essentially anaerobic metabolism of the nucleus[28, 41]. It might well be that the nucleolar orthophosphate is the precursor of RNA in the cell; the synthesis of RNA is made more efficient by the coexistence in the nucleoli of both orthophosphate and a very active RNA fraction (*cf.* ^{32}P uptake and RNA synthesis in *Amoeba* cell fragments[38, 42, 43]). The experiments presented here suggest that the final concentration of orthophosphate is greater in the nucleolus than in the cytoplasm. The question of how much of the total orthophosphate of the whole cell is present in the nucleoli—we may say within the nucleus—must be tested experimentally in several organisms and under different conditions. In *Acetabularia* quantitative determinations showed that not only RNA is concentrated in the nucleated fragment (rhizoide) but also that the total phosphorus is about twice as high as that of the non-nucleated stem[43, 44]. It would be interesting to investigate the distribution of orthophosphate in this large unicellular alga.

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REFERENCES

[1] C. J. Tandler, *J. Histochem. Cytochem.*, 4 (1956) 331.
[2] C. J. Tandler, *J. Histochem. Cytochem.*, 5 (1957) 489.
[3] C. J. Tandler, *Exptl. Cell Research*, 14 (1958) 408.
[4] C. S. Hanes and F. A. Isherwood, *Nature*, 164 (1949) 1107.
[5] S. Burrows, F. S. M. Grylls and J. S. Harrison, *Nature*, 170 (1952) 800.
[6] F. P. W. Winteringham, P. M. Bridges and G. C. Hellyer, *Biochem. J.*, 56 (1955) 13.
[7] Ivor Smith, *Chromatographic Techniques. Clinical and Biochemical Applications*, William Heinemann, Medical Books Ltd., London and Pitman Press, Bath, 1958.
[8] J. B. Jepson and I. Smith, *Nature*, 172 (1953) 1100.
[9] G. Toennies and J. J. Kolb, *Anal. Chem.*, 23 (1951) 823.
[10] G. Brante, *Nature*, 163 (1949) 651.
[11] H. T. Gordon, *Science*, 128 (1958) 414.
[12] E. J. King, *Biochem. J.*, 26 (1932) 292.
[13] R. S. Russell, F. K. Sanders and O. N. Bishop, *Nature*, 163 (1949) 639.
[14] T. A. Geissman and E. Hinreiner, *Botan. Rev.*, 18 (1952) 77.
[15] W. E. Trevelyan, D. P. Procter and J. S. Harrison, *Nature*, 166 (1950) 603.
[16] F. Feigl, *Spot Tests: I. Inorganic Applications.* Elsevier Publishing, Co., Amsterdam, 1954.
[17] T. B. Gage, C. D. Douglass and S. H. Wender, *Anal. Chem.*, 23 (1951) 1582.
[18] U. Khoo and H. T. Stinson, *Proc. Natl. Acad. Sci. U.S.*, 43 (1957) 603.
[19] S. A. Brooks, J. C. Lawrence and C. R. Rickets, *Biochem. J.*, 73 (1959) 566.
[20] S. E. Arney, *Biochem. J.*, 33 (1939) 1078.
[21] P. O. P. Ts'o and C. S. Sato, *Exptl. Cell Research*, 17 (1959) 227.
[22] J. Brachet in *Les Facteurs de la Croissance Cellulaire. Exposés actuels de biologie cellulaire*, Masson et Cie., Paris, 1956.
[23] J. Brachet, *Biochim. Biophys. Acta*, 19 (1956) 583.
[24] F. E. Fritsch, *The Structure and Reproduction of the Algae.* Vol. I, Cambridge University Press, 1935.
[25] W. J. V. Osterhout, *J. Gen. Physiol.*, 29 (1945) 73.
[26] W. J. V. Osterhout, *J. Gen. Physiol.*, 35 (1951) 519.
[27] D. Mazia and D. M. Prescott, *Science*, 120 (1954) 120.
[28] J. Brachet, *Biochemical Cytology*, Academic Press, New York, 1957.
[29] W. S. Vincent, *Internatl. Revue Cytol.*, 4 (1955) 269.
[30] J. H. Taylor, R. Mc Master and M. F. Caluya, *Exptl. Cell Research*, 9 (1955) 460.
[31] J. Hämmerling and H. Stich, *Z. Naturforsch.*, 11b (1956) 158, 162.
[32] R. Mc Master and J. H. Taylor, *J. Biophys. Biochem. Cytol.*, 4 (1958) 5.
[33] P. S. Woods and J. H. Taylor, *Lab. Invest.*, 8 (1959) 309.
[34] P. J. Fitzgerald and K. Vinijchaikul, *Lab. Invest.*, 8 (1959) 319.
[35] L. Goldstein and J. Micou, *J. Biophys. Biochem. Cytol.*, 6 (1959) 1.
[36] G. Richter, *Biochim. Biophys. Acta*, 34 (1959) 407.
[37] G. Richter, *Planta*, 552 (1959) 554.
[38] D. M. Prescott, *J. Biophys. Biochem. Cytol.*, 6 (1959) 203.
[39] E. Baltus, *Biochim. Biophys. Acta*, 15 (1954) 263.
[40] G. H. Hogeboom and W. C. Schneider, *J. Biol. Chem.*, 197 (1952) 611.
[41] H. Stern, *Science*, 121 (1955) 144.
[42] D. Mazia and H. Hirshfield, *Science*, 112 (1950) 297.
[43] J. Brachet, *Symp. Soc. Exptl. Biol.*, 6 (1952) 173.
[44] J. Brachet, H. Chantrenne and F. Vanderhaeghe, *Biochim. Biophys. Acta*, 18 (1955) 544.

TRANSAMINATIONS NON-ENZYMATIQUES DU PYRIDOXAL PHOSPHATE ET DU PYRIDOXAMINE PHOSPHATE À TEMPÉRATURE PHYSIOLOGIQUE ET EN PRÉSENCE DE MÉTAUX

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SUMMARY

Non enzymic transaminations of pyridoxal phosphate and of pyridoxamine phosphate at physiological temperature and in the presence of metals

Non-enzymic transaminations of pyridoxal phosphate with aminoacids and of pyridoxamine phosphate with ketoacids have been studied at $+37°$. Copper and other metals strongly catalyze these reactions as well as the intermediary formation of chelates, which have been separated by paper chromatography. The absorption spectra of the isolated chelates and the spectral composition of the light emitted by u.v. fluorescence have been obtained.

Under experimental conditions where initial concentrations of ketoacids or aminoacids are equal to, or one hundred times higher than, the concentrations of pyridoxamine phosphate or pyridoxal phosphate, the chelates formed in the presence of copper at neutral pH are relatively stable. At pH 5.0, however, the reactions tend to proceed further, with partial decomposition of the chelates and non-enzymic transaminations; these are faster and more complete from pyridoxamine phosphate to pyridoxal phosphate than in the reverse direction.

The structure of the intermediary chelates and the influence of the two tautomeric forms, aldimine and cetimine, on their spectrophotometric properties are discussed.

INTRODUCTION

Le présent travail a été entrepris dans le cadre de recherches sur le mécanisme d'action des enzymes à phosphate de pyridoxal et, plus particulièrement, de la 4-aspartique décarboxylase de *Desulfovibrio desulfuricans*[1, 2]. Des observations qui seront rapportées ailleurs ont montré, en effet, que la réactivation remarquable de cette décarboxylase bactérienne par de très faibles concentrations de céto-acides est due à un processus non-enzymique de transamination, convertissant une forme inactive du coenzyme en sa forme active, aldéhydique.

Les transaminations non-enzymatiques du pyridoxal, de la pyridoxamine et de

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Abréviations: PAL, pyridoxal; PALP, phosphate de pyridoxal; PAMP, phosphate de pyridoxamine; AA, amino acides; CA, céto-acides.

leurs dérivés phosphorylés ont d'abord été observées à +100° par Snell et son groupe[3,4], qui ont étudié l'action catalytique des métaux sur ces réactions. Par la suite, Blakley[5], Metzler[6] et Matsuo[7] ont démontré spectrophotométriquement la formation intermédiaire de bases de Schiff et Christensen[8] a précisé la structure moléculaire et les propriétés physico-chimiques des complexes formés entre le pyridoxal, les amino acides et les métaux. Fasella *et al.*[9] ont séparé par chromatographie les complexes métalliques de phosphate de pyridoxal et d'amino acides produits à +100° en milieu aqueux et ont souligné la forte fluorescence de ces composés. Récemment, enfin, King et Lucas[10] ont étudié, en fonction du pH et de l'amino-acide entrant dans leur constitution, la stabilité des bases de Schiff obtenues en présence et en l'absence de métaux.

Les expériences rapportées ici ont eu pour but de détérminer comment et dans quelle mesure les transaminations non-enzymatiques du phosphate de pyridoxal et du phosphate de pyridoxamine s'effectuent dans les conditions physiologiques où la 4-aspartique décarboxylase est activée par les céto-acides, c'est à dire à +37° et à pH 5.0.

MATÉRIEL ET MÉTHODES

Spectrophotométrie

Les spectres d'absorption des mélanges réactionnels ont été relevés avec un spectrophotomètre Jobin et Yvon, dans des cuves en quartz de 1 cm.

Le spectre de la lumière émise par fluorescence a été enregistré perpendiculairement à un faisceau de lumière de Wood, par un monochromateur muni d'une chambre photographique, et les intensités relevées sur les clichés avec un microphotomètre enregistreur ont été ramenées à une échelle linéaire en longueur d'onde.

Chromatographie

Les mélanges réactionnels ont été chromatographiés sur papier Whatman No. 1 (méthode descendante), avec le mélange[11] butanol tertiaire–acide formique à 99 %–eau (70:15:15). Après développement et séchage des chromatogrammes, les amino acides et le PAMP ont été localisés par la ninhydrine et le PALP et les complexes métalliques des bases de Schiff par leur fluorescence à l'u.v.

Les complexes ont été séparés à partir des chromatogrammes par élution à +32° avec du tampon acétate 0.05 *M* à pH 5.0.

Produits employés

Le PALP utilisé nous a été gracieusement procuré par le Dr. S. Blau (Laboratoires Roche), à qui nous exprimons nos vifs remerciements. Le PAMP était un produit commercial de la California Corporation et l'acide α-céto-glutarique un produit de la Nutritional Biochemicals Corp. Le pyruvate et les amino acides (L-α-alanine et acide L-glutamique) étaient des produits commerciaux Hoffmann-La Roche.

RÉSULTATS EXPÉRIMENTAUX

Dans une série d'expériences préliminaires, l'influence de divers métaux sur les transaminations non-enzymatiques a été étudiée à pH 5.0, avec des mélanges réactionnels contenant du PAMP et une concentration environ 50 fois plus élevée d'α-cétoglutarate. La formation de PALP était estimée par mesure de la densité optique à

388 mμ, en présence de soude 0.1 *N*, conditions où l'absorption de ce composé est maximum.

Les résultats obtenus (Tableau I) montrent que la réaction ne se produit pas en l'absence de métaux et qu'elle est catalysée sensiblement de la même façon par le fer et par le cuivre. Le zinc et le magnésium sont, par contre, inactifs.

Les spectres relevés dans ces systèmes après 5 h d'incubation à +32° correspondent de façon satisfaisante à celui du PALP libre, caractérisé par ses deux maximums d'absorption à 388 et 330 mμ. Par contre, lorsque les concentrations des réactifs, ou le pH, sont modifiés, les produits de réaction comportent des proportions, en certains cas importantes, de complexes intermédiaires dont la formation et l'influence sur les spectres ont été étudiés d'une part dans les mélanges de PALP et d'amino acides et d'autre part dans les mélanges de PAMP et de céto-acides.

TABLEAU I

INFLUENCE DE DIVERS MÉTAUX SUR LA TRANSAMINATION ENTRE LE PHOSPHATE DE PYRIDOXAMINE ET L' α-CÉTO-GLUTARATE

Conditions expérimentales: Systèmes de 4 ml, en tampon acétate 0.1 *M* (pH 5.0) contenant initialement 1.7 μmoles de PAMP et 75 μmoles d' α-céto-glutarate. Densités optiques mesurées à 388 mμ, en présence de NaOH 0.1 *N*, après 5 h d'incubation des mélanges à +32°.

Additions			A_s *(388 mμ)*
Néant			0.000
Fe^{++}	($FeSO_4$)	$2 \cdot 10^{-3}$ *M*	1.600
		$2 \cdot 10^{-4}$ *M*	0.870
Fe^{+++}	($FeCl_3$)	$2 \cdot 10^{-3}$ *M*	1.800
		$2 \cdot 10^{-4}$ *M*	1.590
Cu^{++}	($CuSO_4$)	$2 \cdot 10^{-3}$ *M*	1.800
		$2 \cdot 10^{-4}$ *M*	1.500
Zn^{++}	($ZnCl_2$)	$2 \cdot 10^{-3}$ *M*	0.051
		$2 \cdot 10^{-4}$ *M*	0.009
Mg^{++}	($MgSO_4$)	$2 \cdot 10^{-3}$ *M*	0.005
		$2 \cdot 10^{-4}$ *M*	0.000

Réactions entre PALP et amino acides

METZLER[6] a constaté en l'absence de métaux que le PAL forme avec les amino acides des bases de Schiff, dont le spectre est caractérisé, à pH neutre ou acide, par un maximum d'absorption situé à 414 mμ et par un autre, moins accusé, à 280 mμ. A pH 7.4, ces bases de Schiff se forment en quantités notables lorsque le rapport des concentrations de l'amino acide et du pyridoxal [AA]/[PAL] est compris entre 10^4 et 10^3. En milieu acide et, notamment, à pH 5.0, leur formation est quantitativement minime, même lorsque le rapport [AA]/[PAL] est encore accru.

Des observations similaires ont été faites par MATSUO[7] avec le PALP, à pH 7.5 et en employant des rapports de concentrations du même ordre que ceux de METZLER. Les bases de Schiff formées dans ces conditions ont également deux maxima d'absorption à 400–415 mμ et à 278 mμ.

En nous plaçant dans les conditions décrites par ces auteurs nous avons obtenu des résultats entièrement concordants avec les leurs, c'est à dire la formation de bases de Schiff dont le maximum d'absorption est déplacé vers les grandes longueurs d'onde par rapport à celui du PAL et du PALP. Nous avons vérifié, d'autre part, que si le rapport [AA]/[PALP] est abaissé à 100:1, la formation des bases de Schiff à pH 7.5 se traduit seulement par un déplacement lent et progressif du pic du PALP vers les grandes longueurs d'onde et sa stabilisation, au bout de 4 h environ, à 395 mμ. A pH 5.0, la modification du spectre est encore plus lente et moins marquée. Enfin, lorsque le rapport [AA]/[PALP] est équimoléculaire, on ne constate pratiquement aucune modification d'amplitude ni déplacement du pic d'absorption du PALP, qui demeure fixe, à 388 mμ.

L'addition de cuivre dans les systèmes où le rapport [AA]/[PALP] est compris entre 100:1 et 1:1, entraîne par contre des modifications spectrales extrêmement accusées, qui correspondent à la formation de complexes intermédiaires et, dans certaines conditions, à la formation de PAMP libre, par transamination. Ces réactions ont été étudiées à pH 5.0 (tampon acétate 0.1 M), en présence d'une concentration fixe de $CuSO_4$ ($2 \cdot 10^{-4}$ M). La concentration de PALP, comprise entre 3 et $4 \cdot 10^{-4}$ M, était déterminée exactement d'après le coefficient moléculaire d'absorption de ce composé à 388 mμ et à pH 5.0 ($\varepsilon_M = 5{,}200$). L'amino acide (L-α-alanine ou L-glutamate) était ensuite ajouté à concentration égale ou 100 fois supérieure à celle du PALP. Les mélanges réactionnels étaient incubés à +37° et leurs spectres relevés à intervalles de temps, entre 260 et 460 mμ, en prenant comme blanc un mélange témoin contenant tous les réactifs sauf le PALP.

Dans le cas des mélanges équimoléculaires d'alanine et de PALP, on constate (Fig. 1A) un déplacement progressif du maximum d'absorption vers les courtes longueurs d'onde, et sa stabilisation, au bout de 24 h environ, à 357–360 mμ. Le nouveau pic ne présente pas d'inflexion vers 325 mμ, ce qui indique qu'il ne s'est pas formé de PAMP. Il correspond à un mélange de PALP restant et d'un complexe intermédiaire, qui a été isolé chromatographiquement comme il sera précisé plus loin.

L'absorption du complexe isolé est maximum à 355 mμ et négligeable à 410 mμ, tandis qu'à cette dernière longueur d'onde celle du PALP représente 63.8 % de son maximum. On peut ainsi calculer que la concentration restante du PALP dans le

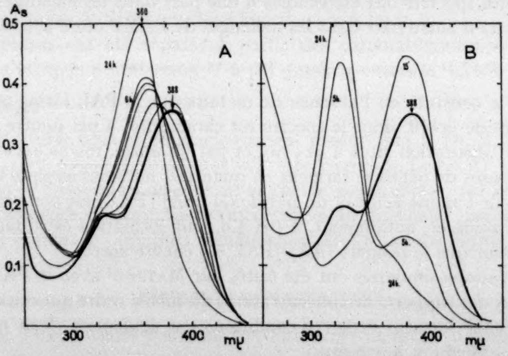

Fig. 1. Spectres d'absorption des mélanges L-α-alanine–PALP–Cu^{++} (pH 5.0; température, 37°). (A) Alanine et PALP à concentrations équimoléculaires ($2.7 \cdot 10^{-4}$ M); (B) alanine 100 fois plus concentrée. Les spectres initiaux sont tracés en trait plus épais.

mélange considéré représente 19.5 % de sa concentration initiale. Comme tout le PALP disparu est engagé dans le complexe, il est également possible, en soustrayant de la densité optique à 355 mμ l'absorption, à cette longueur d'onde, du PALP restant, de déterminer l'absorption propre du complexe et son coefficient moléculaire.

Lorsque la concentration initiale de l'alanine est 100 fois supérieure à celle du PALP (Fig. 1B), le spectre relevé après incubation montre la présence simultanée de PALP, de complexe et de PAMP libre, formé par transamination. Le pic qui culmine vers 380 mμ et dont l'amplitude décroît en cours d'incubation correspond au mélange du complexe et du PALP restant. La concentration du PALP et l'absorption à 355 mμ due au complexe peuvent être calculées comme il vient d'être dit, même en présence

TABLEAU II

COMPOSITION DES MÉLANGES RÉACTIONNELS APRÈS INCUBATION À pH 5.0

Mélanges		*Temps de réaction (h)*	*PALP (%)*	*PAMP (%)*	*Complexe (%)*	*εM du complexe*
			Restant	*Formé*		
Alanine–PALP	1:1	5	66	0	34	8,987
	1:1	24	19.5	0	80.5	4,000
	100:1	5	20	42.8	37	2,644
	100:1	24	6.8	55.9	37.3	4,241
Glutamate–PALP	1:1	5	92.6	6.2	1.1	—
	1:1	24	68	9.4	22.6	2,681
	100:1	5	53	24.3	22.5	2,600
	100:1	24	11.7	59.8	28.5	1,893
			Formé	*Restant*		
Pyruvate–PAMP	1:1	4.5	7.3	41.6	46.9	9,560
	100:1	5	88.8	0	11.2	9,750
α-cétoglutarate–PAMP	1:1	4.5	20.6	70	8.4	9,621
	100:1	5	88.9	7	4	10,500

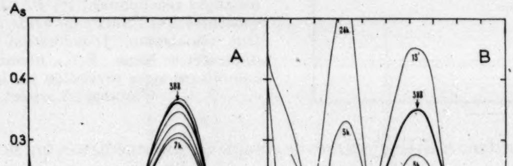

Fig. 2. Spectres d'absorption des mélanges acide L-glutamique–PALP–Cu^{++} (pH 5.0; température, 37°). (A) acide glutamique et PALP à concentrations équimoléculaires; (B) acide glutamique 100 fois plus concentré. Les spectres initiaux sont tracés en trait plus épais.

de PAMP, dont l'absorption à 355 mμ est nulle. La formation de PAMP libre s'exprime par l'apparition progressive d'un nouveau pic à 325 mμ et peut être déterminée quantitativement d'après le coefficient moléculaire du PAMP à pH 5.0 ($\varepsilon_{M}^{325\,m\mu} =$ 10,200), après soustraction des absorptions du complexe et du PALP restant à cette longueur d'onde. Par différence, on obtient la concentration du complexe dont on peut ainsi calculer le coefficient moléculaire d'absorption. Les résultats de ces calculs montrent (Tableau II) que la transamination est déjà importante au bout de 5 h et qu'en fin d'expérience la quantité de PAMP libre formée représente environ 50 % du PALP initial. Ils montrent, d'autre part, que la concentration relative du complexe demeure sensiblement constante pendant toute la réaction et représente environ 37 % du PALP initial.

Dans les mélanges où l'alanine est remplacée par l'acide L-glutamique, on relève des modifications spectrales comparables (Fig. 2A et B). Avec cet amino acide le complexe se forme toutefois plus lentement et est moins stable, ce qui se traduit par une transamination moins complète et par une moindre accumulation du complexe intermédiaire en cours de réaction (Tableau II).

La chromatographie sur papier des mélanges réactionnels (Fig. 3) confirme la formation de PAMP libre dans les mélanges [AA]/[PALP] 100:1. Elle montre en outre la présence dans tous les systèmes de complexes intermédiaires qui se manifestent par une tache fluorescente à l'u.v. (R_F, 0.35), nettement séparée des taches du PAMP (R_F, 0.16) et du PALP (R_F, 0.29). La fluorescence des complexes a une teinte bleue très différente de la fluorescence verte du PALP. A la concentration où ce dernier se trouve dans les mélanges réactionnels, sa fluorescence est d'ailleurs trop faible pour être détectée sur les chromatogrammes. Les complexes ne se colorent pas par la ninhydrine, ce qui indique que la fonction α-aminée de l'amino acide participe à la liaison chimique avec le PALP.

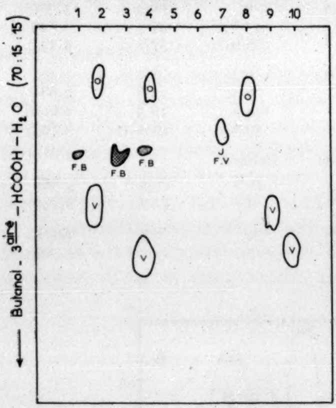

Fig. 3. Chromatographie sur papier des mélanges d'amino acides et de PALP. Systèmes réactionnels: (1) acide L-glutamique–PALP 1:1; (2) acide L-glutamique–PALP 100:1; (3) L-α-alanine–PALP 1:1; (4) L-α-alanine–PALP 100:1; Témoins: (5) α-céto-glutarate; (6) PALP à la même concentration que dans les mélanges réactionnels; (7) PALP 10 fois plus concentré; (8) PAMP; (9) acide L-glutamique; (10) L-α-alanine. J: coloration jaune; F.B.: fluorescence bleue; F.V.: fluorescence verte; colorations après révélation par la ninhydrine: O: orange; V: violet.

Le complexe alanine–cuivre–PALP a été isolé par élution sélective des chromatogrammes. Son spectre d'absorption (Fig. 4) est identique à celui du complexe formé dans la réaction inverse, c'est à dire dans les mélanges de pyruvate, de cuivre et de

PAMP, et comporte un pic unique et symétrique, culminant à 355 mμ. La composition spectrale de la lumière émise par fluorescence est représentée sur la Fig. 5.

A pH 7.5, le cuivre catalyse également la formation de complexes, mais ceux-ci sont beaucoup plus stables qu'à pH 5.0 et on ne constate pas de transamination, même dans les mélanges où les rapports [AA]/[PALP] sont de 100:1. One ne constate pas, en effet, l'apparition du pic caractéristique du PAMP libre, à 325 mμ, mais seulement un déplacement du pic du PALP vers les courtes longueurs d'onde et l'effacement de son inflexion à 350 mμ. Ces modifications sont semblables à celles que MATSUO[7] a relevé à pH neutre et en présence de métaux.

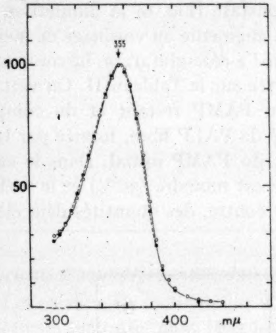

Fig. 4. Spectres d'absorption des complexes isolés par élution des chromatogrammes. ——— : complexe formé dans un mélange équimoléculaire de pyruvate et de PAMP; ----- : complex formé dans un mélange équimoléculaire d'α-alanine et de PALP.

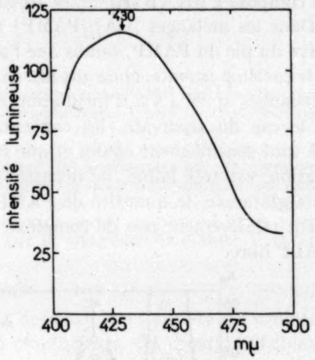

Fig. 5. Spectre de la lumière émise par fluorescence (complexe formé en présence de cuivre dans un mélange équimoléculaire de pyruvate et de PAMP, et séparé par chromatographie). Les intensités lumineuses sont éxprimées en unités arbitraires.

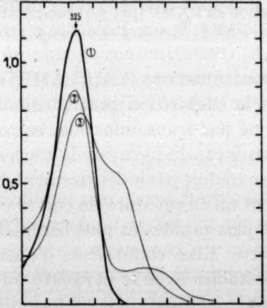

Fig. 6. Spectres d'absorption des mélanges équimoléculaires de céto-acides et de PAMP ($4.4 \cdot 10^{-4}$ *M*) (pH 5.0; température, 37°; temps de réaction: 4.5 h). (1) spectre initial du PAMP; (2) système contenant l' α-céto-glutarate; (3) système contenant le pyruvate.

Réactions entre PAMP et céto-acides

Les réactions inverses, entre PAMP et céto-acides (pyruvate et α-céto-glutarate) ont été étudiées dans des conditions analogues, c'est à dire à +37° et à pH 5.0, en présence de $CuSO_4$ $2 \cdot 10^{-4}$ *M*. Les concentrations initiales du PAMP étaient comprises entre 3 et $4 \cdot 10^{-4}$ *M* et déterminées d'après le coefficient moléculaire d'absorption de ce composé à pH 5.0 ($\varepsilon_{M}^{325\,m\mu} = 10{,}200$).

Dans les mélanges [CA]/[PAMP] 1:1, on constate (Fig. 6) la diminution progressive du pic du PAMP, tandis que l'absorption augmente au voisinage de 360 mμ, dans le cas du pyruvate, et de 385 mμ dans le cas de l'α-céto-glutarate. La composition des mélanges après 4.5 h d'incubation est rapportée sur le Tableau II. On voit que, dans le cas du pyruvate, les concentrations du PAMP restant et du complexe formé sont sensiblement égales et que la quantité de PALP libre, formée par transamination, est très faible, ne dépassant pas 7 % du PAMP initial. Dans le cas de l'α-céto-glutarate, la quantité de PAMP disparue est moindre (30 %) et le mélange contient relativement peu de complexe mais, par contre, des quantités déjà élevées de PALP libre.

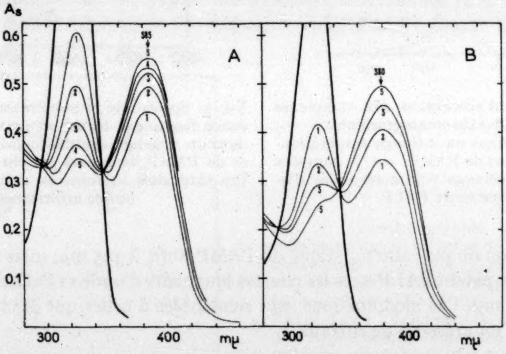

Fig. 7. Spectres d'absorption des mélanges contenant une concentration initiale en céto-acides 100 fois supérieure à la concentration en PAMP (pH 5.0; température, 37°). (A) α-céto-glutarate–PAMP; (B) pyruvate–PAMP. Spectres relevés à intervalles d'une heure.

Lorsque le rapport des concentrations [CA]/[PAMP] est de 100:1 (Fig. 7A et B), on observe au bout de 5 h la disparition pratiquement complète du PAMP. La quantité de PALP libre, formé par transamination, est sensiblement la même avec les deux céto-acides, mais dans le cas du pyruvate la concentration finale du complexe est un peu plus élevée, ce qui se traduit par le déplacement du pic du PALP vers 380 mμ.

Ces observations montrent qu'en présence de cuivre et à pH 5.0, les transaminations non-enzymatiques sont plus rapides et plus complètes dans le sens PAMP ⟶ PALP que dans le sens inverse. Elles confirment, d'autre part, que les complexes métalliques formés sont plus stables avec le pyruvate ou l'alanine qu'avec l'α-céto-glutarate ou l'acide glutamique.

Les chromatogrammes des systèmes PAMP–CA (Fig. 8) montrent que les complexes intermédiaires ont la même mobilité que ceux formés dans les mélanges PALP–AA.

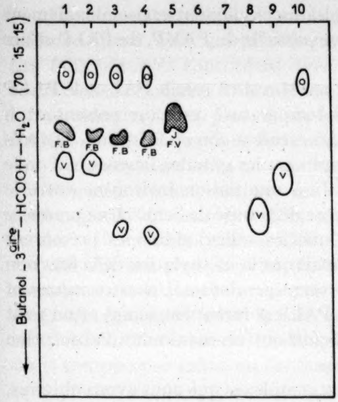

Fig. 8. Chromatographie sur papier des mélanges de céto-acides et de PAMP. Systèmes réactionnels: (1) α-céto-glutarate–PAMP 10:1; (2) α-céto-glutarate–PAMP 100:1; (3) pyruvate–PAMP 10:1; (4) pyruvate–PAMP 100:1; Témoins: (5) PALP concentré; (6) α-céto-glutarate; (7) pyruvate; (8) L-α-alanine; (9) acide L-glutamique; (10) PAMP. J: coloration jaune; F.B.: fluorescence bleue; F.V.: fluorescence verte; colorations après révélation par la ninhydrine: O: orange; V: violet.

Coefficients d'absorption moléculaire des complexes

Les coefficients moléculaires d'absorption des complexes ont été déterminés, par la méthode indiquée, dans tous les mélanges réactionnels. On constate (Tableau II) que leurs valeurs numériques présentent des variations considérables et que, d'une manière générale, elles sont beaucoup plus élevées dans les mélanges où la transamination est quantitativement importante et s'opère dans le sens PAMP ⟶ PALP que dans le sens inverse, ou lorsque se forme un complexe sans transamination concomitante. La signification de ces faits sera discutée plus loin.

DISCUSSION

Nos observations apportent des données nouvelles sur les transaminations non-enzymatiques des dérivés phosphorylés de la vitamine B$_6$. Elles montrent, en particulier, qu'en présence de Cu^{++} et d'autres métaux, ces transaminations s'effectuent non seulement à + 100°, comme l'ont constaté METZLER ET SNELL[3], mais encore à des températures physiologiques, et qu'à pH 5.0 elles atteignent des rendements élevés, même lorsque les rapports initiaux de concentrations [AA]/[PALP] et [CA]/[PAMP] sont faibles. D'autre part, elles confirment et complètent les observations antérieures sur la formation intermédiaire de complexes dans ces réactions, qui peuvent être représentées par le schéma général:

$$\text{PALP} + \text{amino-acide} + \text{Cu}^{++} \leftrightarrows \text{complexe} \leftrightarrows \text{PAMP} + \text{céto-acide} + \text{Cu}^{++}$$

FASELLA *et al.*[9] ont étudié les complexes formés à + 100° et à pH 7.0 entre le PALP, les amino acides et les métaux et les ont séparés chromatographiquement. Ils ont constaté que ces complexes, comme ceux que nous avons obtenus, ont une forte fluorescence bleue et ne se colorent pas par la ninhydrine, ce qui indique que leur structure comporte une liaison imine dans laquelle le groupement $-NH_2$ de l'amino acide est engagé. Cependant, ces auteurs ont constaté au lieu d'un complexe unique deux composés ayant des mobilités chromatographiques différentes et ont relevé, pour celui de ces composés dont ils ont pu étudier le spectre, un maximum d'absorption situé vers 330 mμ. Ces divergences avec nos résultats s'expliquent vraisemblablement par le fait qu'à + 100°, il se produit une hydrolyse partielle du PAMP, du PALP et des complexes eux-mêmes.

Les bases de Schiff formées par METZLER[6] et par MATSUO[7] avec le PAL ou le PALP et les amino acides en l'absence de métaux et à température ordinaire présentent, à pH neutre ou acide, deux maximums d'absorption situés à 280 et à 414 mμ. D'après METZLER ce dernier maximum, fortement déplacé vers les grandes longueurs d'onde par rapport au pic du PAL et du PALP, est dû à une liaison hydrogène entre le groupement 3-hydroxyle du PAL et l'atome d'azote de la base de Schiff. Une première preuve à l'appui de cette interprétation est que les salicyl-aldimines présentent également un maximum d'absorption à 410 mμ, mais que la méthylation de la fonction phénolique ramène à 370 mμ le spectre des imines correspondantes. Un autre argument est qu'en milieu très alcalin, où l'hydroxyle du PAL est fortement ionisé et ne peut contracter une liaison hydrogène, les bases de Schiff ont un maximum d'absorption unique et situé non plus à 414 mμ mais à 360 mμ.

Ces considérations conduisent à attribuer aux complexes que nous avons obtenus, et dont le maximum d'absorption est situé à 355 mμ, la configuration proposée par LONGENECKER ET SNELL[12] et par CHRISTENSEN[8], c'est à dire une structure dans laquelle le cuivre est combiné avec les deux hydroxyles du noyau pyridine et de l'amino acide, ce qui a pour effet d'empêcher l'établissement d'une liaison hydrogène entre l'hydroxyle de la pyridine et l'azote de l'imine. Les deux formes tautomères aldimine (I) et cétimine (II) d'un tel complexe et l'ordre de leurs interconversions au cours des transaminations sont représentées sur la Fig. 9.

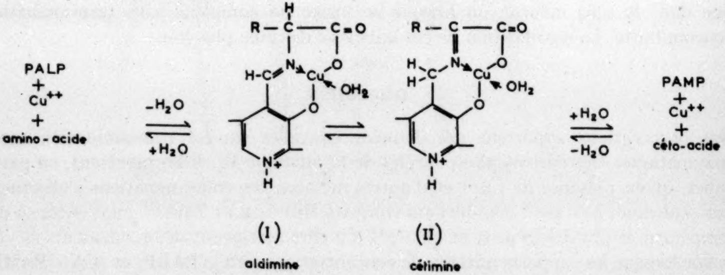

Fig. 9. Structure des complexes intérmédiaires. Pour la clarté de la figure, on a omis de représenter sur le cycle pyridine les substituants 2-méthyle et 5-hydroxy-méthyle phosphate.

En règle générale, la condition structurale nécessaire pour que les dérivés de la vitamine B_6 aient un maximum d'absorption situé entre 330 et 390 mμ est l'existence de deux doubles liaisons conjuguées, l'une sur le carbone substitué en position 4 et l'autre sur le cycle pyridine[13]. En ce qui concerne les complexes, cette condition n'est réalisée que par la structure aldimine et, de ce fait, il est probable que l'absorption à 355 mμ est exclusivement due à cette forme. Dans le cadre de cette hypothèse, les coefficients moléculaires d'absorption qui ont été déterminés expérimentalement (Tableau II) dépendent de l'équilibre entre les deux formes tautomères des complexes et de leurs concentrations relatives, ce qui explique leurs variations suivant les conditions expérimentales.

On doit s'attendre à ce que la forme prédominante du complexe soit, dans un mélange donné, celle qui précède immédiatement les produits finaux de la réaction. Les faits observés concordent avec cette prévision. Ils montrent, en effet, que le coefficient moléculaire d'absorption, et corrélativement la proportion relative de la forme aldimine, sont plus élevés lorsque la transamination s'effectue dans le sens PAMP → PALP que dans le sens inverse.

Les complexes isolés chromatographiquement à partir des mélanges réactionnels où les concentrations initiales des réactifs sont équimoléculaires ont exactement le même spectre d'absorption (Fig. 4), qu'il s'agisse des systèmes amino acide–PALP ou des systèmes céto-acide–PAMP. Cette observation montre que, comme dans le cas des dérivés non-phosphorylés de la vitamine B_6 (voir réf. 14), la forme stable des complexes est la structure aldimine. La plus grande stabilité de cette dernière forme concorde également avec le fait que le coefficient d'absorption du complexe est relativement élevé dans les mélanges équimoléculaires d'alanine et de PALP, mélange où le complexe se forme en l'absence de toute transamination.

RÉSUMÉ

Les transaminations non-enzymatiques du phosphate de pyridoxal avec les amino acides et du PAMP avec les céto-acides ont été étudiées à $+37°$. Le cuivre et d'autres métaux catalysent activement ces réactions et la formation intermédiaire de complexes qui ont été isolés par chromatographie sur papier. Les spectres d'absorption des complexes isolés et la composition spectrale de la lumière qu'ils émettent par fluorescence dans l'u.v. ont été relevés.

Dans les conditions expérimentales employées, c'est à dire lorsque les concentrations initiales du céto-acide ou de l'amino acide sont égales ou 100 fois supérieures à celles du phosphate de pyridoxamine ou du phosphate de pyridoxal, les complexes formés en présence de cuivre et à pH neutre sont relativement stables. En milieu acide, par contre, les réactions tendent à se poursuivre et la décomposition partielle des complexes aboutit à des transaminations non-enzymatiques, qui sont plus rapides et plus complètes dans le sens phosphate de pyridoxamine → phosphate de pyridoxal qu'en sens inverse.

La structure des complexes intermédiaires et l'influence de leurs deux formes tautomères aldimine et cétimine sur leurs propriétés spectrophotométriques sont discutées.

BIBLIOGRAPHIE

[1] J. C. Senez et J. Cattanéo-Lacombe, *Compt. rend.*, 242 (1956) 943.
[2] J. Cattanéo-Lacombe, J. C. Senez et P. Beaumont, *Biochim. Biophys. Acta*, 30 (1958) 458.
[3] J. C. Rabinowitz et E. E. Snell, *J. Biol. Chem.*, 169 (1947) 643.
[4] D. E. Metzler et E. E. Snell, *J. Am. Chem. Soc.*, 74 (1952) 979.
[5] R. L. Blakley, *Biochem. J.*, 61 (1955) 315.
[6] D. E. Metzler, *J. Am. Chem. Soc.*, 79 (1957) 485.
[7] Y. Matsuo, *J. Am. Chem. Soc.*, 79 (1957) 2011 et 2016.
[8] H. N. Christensen, *J. Am. Chem. Soc.*, 79 (1957) 4073.
[9] P. Fasella, H. Lis et N. Siliprandi, *Biochim. Biophys. Acta*, 23 (1957) 417.
[10] H. K. King et N. Lucas, *Biochem. J.*, 72 (1959) 18 P.
[11] R. J. Block et D. Bolling, *The amino-acid composition of proteins and foods*. Analytical methods and results, 2nd ed., C. C. Thomas, Springfield, Illinois, 1951.
[12] J. B. Longenecker et E. E. Snell, *Proc. Natl. Acad. Sci. U.S.*, 42 (1956) 221.
[13] D. E. Metzler et E. E. Snell, *J. Am. Chem. Soc.*, 77 (1955) 2431.
[14] G. L. Eichhorn et J. W. Dawes, *J. Am. Chem. Soc.*, 76 (1954) 5663.

ELECTRICAL ACTIVITY EVOKED BY A SPECIFIC CHEMICAL REACTION

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SUMMARY

The aim of the investigations has been the demonstration that electrical activity, irreversibly blocked by the inhibition of acetylcholinesterase with organophosphorus compounds, may be restored by a specific chemical reaction, viz. by reversing the process with a specific reactivator which displaces the phosphoryl group from the enzyme.

1. Frog Sartorius muscle, dissected into two strips, was exposed to Paraoxon until irreversible block of electrical activity was reached. One strip was then exposed to the reactivator.

2. Pyridine-2-aldoxime methiodide (2-PAM), a powerful reactivator of phosphorylated enzyme, and several more lipid soluble derivatives did not restore electrical activity either in normal or in denervated muscle. Electrical activity could be evoked in strips in which it had been nearly completely abolished, with benzoyl-2- and -4-pyridine aldoxime methiodide. Nine out of eleven experiments were successful.

3. The inhibitor used was not quite suitable: the transition from irreversible block of electrical activity to a severe damage of the muscle, with gross alterations such as rigidity and decoloration, took place rather fast. The multifiber preparation used was for other reasons unfavorable for the purpose of the experiments. It is suggested that with other organophosphorus inhibitors and other types of preparation a still better demonstration would be possible that electrical activity may be evoked by a specific chemical reaction.

4. Frequently, the spike height and amplitude of the muscle, on exposure to either eserine or Paraoxon, were markedly increased before the decrease and eventual abolition. With Paraoxon the effect occurred sometimes in the recovery phase. These effects support the assumption that the rapid increase in sodium conductance during nerve activity is due to the action of acetylcholine and the rapid restoration to the normal level to that of acetylcholinesterase.

INTRODUCTION

The importance of the acetylcholine system for the generation of bioelectric potentials, which propagate impulses in the conducting membranes of nerve and muscle, has

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Abbreviations: PAM, pyridine-2-aldoxime methiodide; PAD, pyridine-2-aldoxime dodecyl iodide; DFP, diisopropylfluorophosphate.

been demonstrated in many ways. The data have recently been summarized and evaluated in a monograph by NACHMANSOHN[1]. One essential point upon which the evidence is based, is the inseparability of conduction from the activity of acetylcholinesterase demonstrated with specific inhibitors on a great variety of conducting tissues. Under no condition has it been possible to dissociate electrical and enzyme activity in any type of conducting tissue: electrical activity invariably stops when the enzyme activity falls to about 20 % of the initial value.

The development of organophosphorus compounds, which are powerful irreversible cholinesterase inhibitors, their increasing application as insecticides, and their potential use as chemical warfare agents ("nerve gas") rendered the mode of the reaction of these compounds with the enzyme of great theoretical and practical interest. The elucidation of the precise mechanism has revealed that these compounds, when reacting with the enzyme, form a phosphorylated enzyme instead of the acetylated enzyme which is the intermediary form in the physiological process[2]. But whereas the acetylated enzyme reacts rapidly with water and leads to acetate and restored enzyme, the phosphoryl group can only be displaced from the enzyme by a chemical reaction[3]. One of the most powerful compounds specifically designed to reactivate the phosphorylated enzyme proved to be PAM[4]. The speed of reactivation is so high that PAM applied to animals proved to be an efficient antidote, especially combined with atropine[5,6].

It appeared of interest to test whether conduction irreversibly blocked by organophosphorus compounds could be restored by the reactivation of phosphorylated enzyme. In the affirmative case it would be a demonstration that the abolished electrical activity can be restored by a highly specific chemical reaction, *i.e.*, the reactivation of acetylcholinesterase. The significance of such an experiment is obvious. PAM is insoluble in lipids and could not be expected to reactivate the enzyme in conducting fibers since it is known that such type of compounds do not affect the conducting membrane[7,8]. Therefore, a modification of its structure consisting in a substitution of the methyl group by a dodecyl group was introduced: 2-PAD, making the compound very lipid soluble. However, when this compound was applied to conducting tissue, it proved to be a strong blocking agent of electrical activity: it depolarizes the conducting membrane and produces muscular contraction outside the synapse by direct action on the conducting membrane, thus acting to some extent as a lipid soluble analogue of acetylcholine[9–14]. In higher concentrations the action is difficult to reverse. Thus, although valuable in other respects, the compound was useless for the specific purpose for which it was originally designed.

In 1959, AXELSON AND THESSLEFF[15] reported an effect of acetylcholine on denervated muscle outside the neuromuscular junction. This ester, as well as other methylated quaternary ammonium compounds, is known to have no effect on the conducting membrane of normal muscle when applied externally. The new results seemed to indicate, therefore, that following denervation an increase in permeability to acetylcholine may take place so that now a fraction of the compound applied may reach the active site.

If denervation facilitates access of acetylcholine to the active sites in the conducting membrane, it may do the same for other quaternary ammonium ions, such as 2-PAM. In that case, reactivation of phosphorylated enzyme within the membrane and restoration of irreversibly blocked electrical activity seemed to be possible.

Experiments testing this assumption showed, however, that the increase in permeability is relatively small and, in any case, not adequate enough to produce the desired result. However, another derivative of PAM, benzoyl PAM, more lipid soluble than PAM but not quite as lipid soluble as PAD, proved to be more successful and the results obtained are described in this paper.

METHODS

Summer stock Rana pipiens were used. About 1 cm of the lumbosacral plexus on one side of the frog was removed under urethane anesthesia. The frogs were kept for one month or more to insure complete denervation before they were used for experiments. Since a change in permeability to various compounds was expected following denervation, the contralateral Sartorius muscle could not be used as control. Each Sartorius was therefore dissected longitudinally into about equal halves and one of these used as the control for the other. This procedure had the additional advantage that the strips were thinner than the whole muscle and the penetration of the compounds was, therefore, facilitated. The strips were kept in oxygenated Ringer for 1 h or more and were then mounted in the experimental chamber. Two platinum electrodes and a Grass stimulator were used for stimulation. Recordings were obtained by external platinum electrodes, a Grass AC Preamplifier and an oscilloscope. The records were photographed with a Grass camera. The composition of Ringer solution was 119 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 2.5 mM $NaHCO_3$. The pH was frequently checked and kept within 7.3–7.5 range except when working with eserine, when a slightly more alkaline pH (about 7.8) was used. All experiments were done at room temperature.

The organophosphorus compound used was Paraoxon. About 30 experiments with this compound alone were performed in order to obtain the optimal concentration and the appropriate time of exposure needed to produce irreversible block of action potential without leading to severe damage to the muscle.

Reactivators tested were PAM, isonicotinoylformaldoxime methiodide, isonicotinoylformaldoxime methiodide, isonicontinoylformaldoxime (tertiary), 2-benzoylpyridine oxime methiodide and 4-benzoylpyridine oxime methiodide. All these compounds were synthesized by Dr. S. GINSBURG. Paraoxon was prepared by Dr. A. GOLD. The authors are greatly indebted to both.

RESULTS

Effects of eserine and Paraoxon

Eserine in concentrations of 1 to 7 μmoles/ml abolished the electrical activity, the response to direct stimulation, within 10 to 40 min. This inhibition was readily reversible. Although some difference was observed in the response of normal and denervated muscle, it was not striking. On several occasions block of electrical activity by eserine was preceded by a significant increase in the amplitude of the action potential (Fig. 1).

Paraoxon in 4 μmoles/ml also abolished electrical activity of normal and denervated muscle, but much faster than eserine. On short exposure to Paraoxon the inhibition was at first reversible, as described with other irreversible inhibitors, such as for instance DFP, on a great variety of nerve fibers[1]. Irreversible inhibition of electrical activity was obtained with either increased concentration of the compound or by longer exposure. Too long exposure led to severe and irreparable damage grossly indicated by decoloration, contracture and rigidity of the muscle. Optimum concentration of Paraoxon which blocked conduction without noticeable damage was found to be about 5–6 μmoles/ml and the duration of the exposure 8 to 15 min. The sensitivity to Paraoxon varied, however, greatly from muscle to muscle, while the two sections of the same muscle usually behaved in essentially the same way. Again, no marked difference was observed between the normal and the denervated muscle.

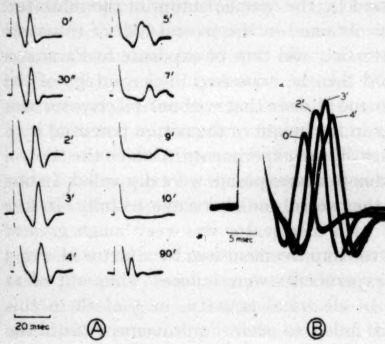

Fig. 1. Increase of the height of the action potential in a frog sartorius muscle on exposure to eserine. The muscle was exposed to $7.2 \cdot 10^{-3}$ M eserine salicylate for 10 min and 30 sec at pH 7.8. A. Records shown were taken at 0 time and after exposure at times indicated in the Figure. Stimulus artifact precedes action potential. Electrical activity was virtually abolished after 10 min. Partial recovery after 90 min. B. Same records superimposed. In the following figures only the superimposed records will be shown.

An increase in spike height and amplitude of muscle exposed to Paraoxon was observed in 13 different experiments, either during the initial period of exposure —quite similar to the eserine effect—or during the recovery phase in those muscles which were exposed to concentrations insufficient for blocking electrical activity irreversibly (Figs. 2 and 3).

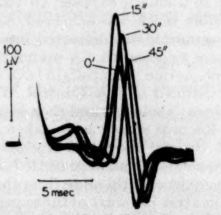

Fig. 2. Increase of spike height in a frog sartorius muscle on exposure to Paraoxon (1.5 mg/ml). Records shown (superimposed) were taken at 0 time and 15, 30 and 45 sec after exposure.

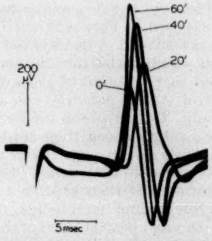

Fig. 3. Increase of the height of action potentia in denervated frog Sartorius muscle during recovery, after the muscle had been exposed to Paraoxon (1 mg/ml) for 10 min. Records shown (superimposed) were taken at 0 time and 20, 40 and 60 min after the start of the experiment.

Effect of antidotes on electrical activity of muscles exposed to Paraoxon

After a series of experiments with Paraoxon alone it became clear that it was only a very short step from an irreversibly inhibited preparation, which remained viable, to one which was irreparably damaged. It appeared that the relatively best procedure was that of producing a condition in which the irreversible inhibition was not yet complete but would permit on washing with Ringer's solution a small spontaneous recovery of electrical activity indicating that with this concentration and time of exposure to Paraoxon the muscle remained in a relatively fair condition while most of the electrical activity remained suppressed by the specific action of the inhibitor. The improvement of the electrical activity obtained in the second half of the same muscle inhibited with the identical concentration and time of exposure to Paraoxon but exposed thereafter to an antidote could then be expressed in percentage of the control recovery. An improvement of 50 to 100 % over that without reactivator was considered a mild success, while an increase in the height of the action potential by a factor of 3 or more was accepted as significant. Those experiments in which the control muscle clearly showed irreparable damage due to overexposure were discarded. In one the exposure was not long enough so that the control muscle recovered fully. In five the recovery in the muscle strips treated with reactivator was very much greater than in the controls (Figs. 4 and 5). In two the improvement was by a factor of 2 to 3 and in two by a factor less than 2. Two experiments were failures. Thus out of 11 experiments 9 have shown improvement of electrical activity, in 5 of them this improvement was excellent, while only 2 failed to show improvement with the reactivator.

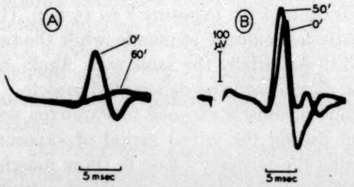

Fig. 4. Reappearance of electrical activity by the action of benzoyl-pyridine oxime methiodide in a muscle exposed to Paraoxon until irreversible block was achieved. A denervated frog sartorius muscle was dissected into two strips, both were exposed to 1.5 mg/ml of Paraoxon for 8 min. A, control. Action potential was abolished within 3.5 min. The strip was washed 3 times with Ringer's solution and then kept in that solution. Records shown were taken at 0 and 60 min. B, second strip was washed 3 times with Ringer's solution and then kept in 10^{-2} *M* 2-benzoylpyridine oxime methiodide from the 9th to the 45th min after the start of the experiment. Records shown were taken at 0 and 50 min. Records are superimposed.

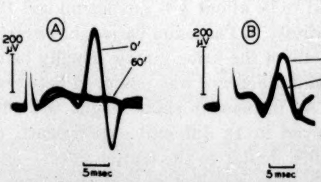

Fig. 5. Reappearance of electrical activity by the action of benzoyl-pyridine oxime methiodide in a muscle exposed to Paraoxon until irreversible block was achieved. A normal frog sartorius muscle was dissected into two strips; both were exposed to 1.5 mg/ml of Paraoxon for 8 min. Action potentials in both strips disappeared within 2 min. A. Control. Washed twice with Ringer's solution and then kept in that solution. Records shown were taken at 0 and 60 min. B. Second strip was washed twice with Ringer's solution and kept in 10^{-2} *M* 2-benzoylpyridine oxime methiodide from the 9th to the 30th min after the start of the experiment, then returned to Ringer's solution. Records shown were taken at 0 and 60 min.

DISCUSSION

Although extensive investigations have established that electrical activity in axons fails when acetylcholinesterase activity falls below a certain level, about 20 % of the initial value, the result was challenged by DEL CASTILLO AND KATZ[16] on the basis that the concentrations used are "unconventionally" high when compared with the concentrations used at synaptic junctions. The authors implied an unspecific action of the inhibitors. The high concentrations are, however, necessary because axons are surrounded by structural barriers. Moreover, the assumption of an unspecific effect is difficult to reconcile with the various demonstrations that different types of chemical compounds used at different concentrations under a variety of conditions invariably block conduction at the same level of esterase activity. The time of exposure required may differ greatly but the percentage of activity essential for conduction is the same. Recently, DETTBARN applied eserine to single Ranvier nodes of single fibers of frog sciatic nerve and found the effectiveness of this compound to block conduction increased by a factor of 1000 (see ref. 12). In this preparation, as recent electron microscope pictures have revealed, the conducting membrane is only poorly protected by a thin and apparently porous structure[17].

Obviously, the inseparability of electrical and enzyme activity would gain strong additional support by the demonstration that irreversibly blocked electrical activity may be evoked again by the specific reactivation of acetylcholinesterase. PAM has been designed to reactivate specifically phosphorylated acetylcholinesterase. It has been shown to have molecular complementarity to the phosphorylated enzyme[18]. For other phosphorylated esterases PAM is a relatively poor reactivator. This compound would, therefore, be suitable for the demonstration desired except for its lipid insolubility preventing it from reaching the active site. The expectation that permeability to lipid insoluble quaternary compounds would sufficiently increase in denervated muscle fibers did not materialize. Among the modifications of PAM making the molecule more lipid soluble, so far only benzoyl 2- and 4-PAM proved to be effective. It was possible to evoke good electrical activity after exposure to the reactivator in muscle fibers in which electrical activity was nearly completely and irreversibly abolished.

The experiments are not yet fully satisfactory. Out of 11 experiments 2 were failures. It would be desirable to obtain experimental conditions where completely and irreversibly blocked fibers would regain normal electrical activity. Various factors are unfavorable in the experiments performed. The material used is a multifiber preparation. Both simultaneous inhibition and simultaneous reactivation of all the conducting membranes appear unlikely since the penetration is relatively slow and the compounds applied will reach the membranes of the various fibers at different periods of time. It is easy to see why this type of experimental material is unfavorable. The effect of the inhibitor should be rather fast because a prolonged exposure frequently induces secondary changes which may be irreversible even if the enzyme is reactivated. It would be desirable to have greater leeway between the onset of irreversible inhibition and severe secondary alterations. Low concentrations of Paraoxon did not produce irreversible inhibition for quite a long period of time. The higher concentrations used left only a very short period between irreversible block and severe secondary damage such as decoloration and rigidity. Other organophosphorus compounds have not been tested; some of them may be much more favorable for the aim of these experiments. Obviously, therefore, investigations with more favorable material and more favorable inhibitors are desirable and are planned. However, in spite of all the improvements possible, the data presented have shown that electrical activity in fibers in which it was virtually irreversibly abolished, may be restored by means of a compound known to reactivate specifically the phosphorylated enzyme.

Another significant result of the observations reported is the increase in spike height and amplitude in the muscle fibers exposed to inhibitors of acetylcholinesterase. This is exactly what would be expected from theory: if the action of acetylcholine on the receptor protein increases sodium conductance and the rapid hydrolysis of the ester by acetylcholinesterase permits the return of sodium conductance to its resting value, a decreased rate of acetylcholine removal should lead to an increased amplitude. However, such an effect may be easily veiled by a series of factors. The enzyme is present in excess. As long as there is an excess the rate of hydrolysis should be adequate for a normal rate of conductance changes. If the concentration falls below a certain level, conduction rapidly fails. The range between normal, adequate concentration of enzyme and that incompatible with conduction seems to be very narrow and this may make it in many cases very difficult to observe the phase of increased spike height and amplitude which may be very short and transitory. It will be particularly difficult to obtain this effect on multifiber preparations in which this effect may be absent due to the lack of synchronization of the electric responses of individual fibers.

Recently, DETTBARN observed an increase of spike height and amplitude on Ranvier nodes of a single frog sciatic nerve fiber exposed to either eserine or Paraoxon[12]. There, the conditions are favorable. Unexpectedly we found the same effect on the muscle strips exposed to either eserine or Paraoxon. The increase is occasionally quite marked before the decrease of electrical activity starts. With Paraoxon the effect appeared sometimes during recovery. The return of electrical activity usually takes place at a much slower rate than its disappearance. This may favor a more graded restoration in which the relatively short range between too much and too little enzyme in which the effect takes place, may be sometimes found more easily than during the initial phase.

ACKNOWLEDGEMENTS

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REFERENCES

[1] D. NACHMANSOHN, *Chemical and Molecular Basis of Nerve Activity*, Academic Press, New York, 1959.
[2] I. B. WILSON AND F. BERGMANN, *J. Biol. Chem.*, 185 (1950) 479.
[3] I. B. WILSON, *J. Biol. Chem.*, 190 (1951) 111.
[4] I. B. WILSON AND S. GINSBURG, *Biochim. Biophys. Acta*, 18 (1955) 168.
[5] H. KEWITZ AND I. B. WILSON, *Arch. Biochem. Biophys.*, 60 (1956) 261.
[6] H. KEWITZ, I. B. WILSON AND D. NACHMANSOHN, *Arch. Biochem. Biophys.*, 64 (1956) 456.
[7] T. H. BULLOCK, D. NACHMANSOHN AND M. A. ROTHENBERG, *J. Neurophysiol.*, 9 (1946) 9.
[8] M. A. ROTHENBERG, D. B. SPRINSON AND D. NACHMANSOHN, *J. Neurophysiol.*, 11 (1948) 111.
[9] E. SCHOFFENIELS, I. B. WILSON AND D. NACHMANSOHN, *Biochim. Biophys. Acta*, 27 (1958) 629.
[10] W. D. DETTBARN, I. B. WILSON AND D. NACHMANSOHN, *Science*, 128 (1958) 1275.
[11] W. D. DETTBARN, *Biochim. Biophys. Acta*, 32 (1959) 381.
[12] W. D. DETTBARN, *Biochim. Biophys. Acta*, 41 (1960) 377.
[13] L. P. HINTERBUCHNER AND I. B. WILSON, *Biochim. Biophys. Acta*, 31 (1959) 323.
[14] L. P. HINTERBUCHNER AND I. B. WILSON, *Biochim. Biophys. Acta*, 32 (1959) 375.
[15] J. AXELSSON AND S. THESLEFF, *J. Physiol. (London)*, 147 (1957) 178.
[16] J. DEL CASTILLO AND B. KATZ, *Progr. in Biophys. and Biophys. Chem.*, 6 (1956) 121.
[17] J. D. ROBERTSON, in D. NACHMANSOHN, *Molecular Biology*, Academic Press, New York, 1960, p. 87.
[18] I. B. WILSON, S. GINSBURG AND C. QUAN, *Arch. Biochem. Biophys.*, 77 (1958) 286.

Biochim. Biophys. Acta, 44 (1960) 554–560

ISOLATION AND IDENTIFICATION OF THE ACETYLCHOLINE RECEPTOR PROTEIN OF ELECTRIC TISSUE

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SUMMARY

The isolation and identification of the acetylcholine receptor protein has been achieved in the following way:

1. The proteins of electric tissue of *Electrophorus electricus* have been extracted and fractionated with ammonium sulfate.

2. Applying equilibrium dialysis under strict control of pH and ionic strength, a protein component has been found which binds acetylcholine and related tertiary and quaternary nitrogen derivatives such as *d*-tubocurarine (curare), atropine, procaine, Prostigmine and benzoylcholine. The binding strength of these compounds varies greatly.

3. This protein forms a precipitate with curare. The latter compound readily precipitates a variety of macromolecules such as nucleic acids, acidic polysaccharides, and others. But whereas these complexes are readily solublized by dialysis at neutral pH, the receptor protein-curare precipitate can only be solublized by raising the pH to 9. Using curare precipitation, the receptor protein has been isolated in electrophoretically homogeneous form.

4. Identification with the cellular acetylcholine receptor has been achieved by the demonstration of a striking parallelism between the binding strength of the various compounds tested in solution and their effectiveness in affecting electrical activity of the isolated single electroplax prepared from electric tissue. Other macromolecules tested showed binding features distinctly different from those of the receptor protein.

INTRODUCTION

Studies by NACHMANSOHN, WILSON *et al.*[1,2] have contributed greatly to an understanding of the interaction of cholinesterase with acetylcholine and with a variety of inhibitors of the enzyme. These ideas have been extrapolated by NACHMANSOHN[1] to the concept of a "receptor protein", present in the conducting membranes of nerve and muscle as well as at synaptic junctions and considered to have molecular features at the active site similar to those of acetylcholinesterase. This concept has been successfully applied to an understanding of the behavior of a large number of

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Abbreviations: TRIEG, triethiodide of gallamine; ACh, acetylcholine; PAM, pyridinealdoxime methiodide; CPC, cetyl pyridinium chloride; PAD, pyridine aldoxime dodeciodide; TEPP, tetraethyl pyrophosphate; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; DPC, dodecyl pyridinium chloride; BSA, bovine serum albumin.

compounds insofar as their effect on nerve and muscle is concerned, the interpretations being based on interactions with both the enzyme and receptor protein. Thus, the reaction of a number of tertiary and quaternary nitrogen compounds with cholinesterase and their effects on electrical activity of various cells have certain features in common[3,4]. As a further extension from the work on the enzyme, it was postulated that the apparent configurational changes of cholinesterase effected by acetylcholine during hydrolysis[5] might, if paralleled by similar changes in the receptor protein, provide a molecular mechanism for the altered ion permeability associated with nerve conduction[1].

While the notion of a receptor protein is a widely accepted one, direct experimental evidence for its existence in any sort of isolated form has been completely lacking, all data in this field having been obtained exclusively with living cells. A notable attempt to obtain the receptor in solution has recently been made by CHAGAS *et al.*[6,7]. Those workers injected radioactive triethiodide of gallamine—a curare-like substance—into electric eels, extracted material from the electric organ, a tissue known to be rich in receptor, and considered the retention of the radioactivity after exhaustive dialysis of the extract against distilled water as the basis for receptor activity. As has been found[8–10] and will be elaborated on below, a number of macromolecules, such as acidic polysaccharides and nucleic acids, bind curare—and presumably TRIEG—nonspecifically, although such complexes behave in a manner similar to that described by CHAGAS *et al.*, *i.e.*, slow dialyzability of the curare in distilled water, rapid dialysis in dilute salt. Recognizing this fact, these investigators have now concluded that their findings may have uncovered nonspecific receptors for curare or TRIEG rather than the cellular component with which ACh reacts during electrical activity[11]. This interpretation has recently been stressed by CHAGAS at a recent symposium in Rio de Janeiro. In this report, evidence will be presented that a protein is present to a small extent in the extract of electric tissue and muscle which interacts much more strongly with curare. The high affinity of this substance for curare, differentiating it from other curare-binding components, gives rise to the intriguing possibility of a physiological role for this protein.

In pursuing the problem of attempting to isolate the receptor, CHAGAS' procedure has been modified in two important aspects: First, after extracting the electric tissue with dilute buffer, an ammonium sulfate fractionation procedure was undertaken in order to eliminate any non-protein components such as free acidic polysaccharides or nucleic acids present in the extract. Next, equilibrium dialysis has been used under controlled conditions of pH and ionic strength to study the interaction of various neurotropic compounds rather than exhaustive dialysis against distilled water which introduces complications due to DONNAN effect. As a check on the specificity of interaction, binding studies were conducted with bovine serum albumin and chondroitin sulfate. Whenever possible, results in solution were compared with those obtained from studies on single cells of the electric organ, although the difficulties of precise comparison are obvious.

METHODS AND MATERIALS

Preparation of tissue proteins for various studies

The method adopted for obtaining the proteins of the electric tissue in solution is as follows: The main organ of electric tissue was excised from the freshly killed animal and freed as much as possible from muscle, connective tissue, blood, etc. If not used immediately, the tissue was stored at —15° at which temperature it can be kept for a long time with no evidence of decomposition. Extraction of the proteins was carried out as follows: Small portions of the frozen or fresh tissue were cut with scissors, placed in ice cold phosphate buffer at pH 7.5, ionic strength 0.1, containing a small amount of merthiolate and homogenized for about 5 min in a Waring blender. The volume of buffer was 1 to 1.5 ml/g of tissue. The mixture was shaken overnight to complete the extraction after which it was centrifuged at 4000 rev./min at 0° for about 1 h. Coagulated fat, which floats to the surface, was removed by aspiration. The residue was stored at —15° and was used without further treatment. The supernatant was concentrated to about one third of the original volume by pervaporation at 0°; after that it was dialyzed first against running tap water, then against dilute phosphate buffer followed by fractionation of the proteins with ammonium sulfate as described below.*

TABLE I

FRACTIONATION OF ELECTRIC TISSUE EXTRACT WITH AMMONIUM SULFATE

% saturation ammonium sulfate	*% of total protein precipitated*	
	Undiluted extract	*After 5 fold dilution*
20	4	0
30	27	20
40	44	38
50	55	44
60	60	44

Preliminary ammonium sulfate fractionation was carried out as follows: To the undiluted extract containing about 2 % protein and to a 5-fold dilution of the extract varying amounts of saturated ammonium sulfate were added to give 20, 30, 40, 50 and 60 % saturations. After standing overnight in the refrigerator, all solutions were centrifuged and the O.D. of the supernatants determined at 280 mμ. Table I shows (a) that the composition of each fraction depends quite markedly on the protein concentration and (b) that even at 60 % saturation a considerable amount of material remains in the supernatant with more being present at the lower protein concentration. Since a greater number of fractions were obtained at higher protein concentrations (about 2 %), all large scale fractionations were carried out with the tissue extract at about this protein concentration.

In this procedure the saturated ammonium sulfate was added dropwise to the ice cold extract to obtain 30 % saturation, the solution was placed in the refrigerator for about 1 h to complete precipitation after which it was centrifuged at 4000 rev./min for about 1 h. The precipitate was suspended in 30 % ammonium sulfate, recentrifuged and dialyzed first against running tap H_2O, then against phosphate buffer to remove the sulfate. Additional saturated ammonium sulfate was added to the supernatant to 40 % saturation, followed by the above treatment. This was repeated at intervals of 10 % saturation up to the 60 % level, except that with increasing saturation much longer periods of time (up to one day) were required to complete precipitation. 60 % saturation was considered the highest feasible ammonium sulfate concentration for obtaining a precipitate. In another procedure, the ammonium sulfate concentration was adjusted directly to either 30 % or 60 % saturation followed by centrifugation, washing etc. These precipitates are referred to as 30 % and 60 % fractions.

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* Pre-extraction of the tissue with toluene, used in the preparation of cholinesterase to remove mucin[12], did not improve the yield of extractable protein.

In order to determine whether precipitation was complete in the time allotted, an aliquot of each supernatant fraction was removed and placed in the refrigerator for several days. In no instance were additional precipitates observed.

In later procedures, especially for the preparation of 30 % fraction, solid ammonium sulfate was added to the ice cold protein solution with constant stirring to give the desired saturation.

Isolation of receptor protein by curare precipitation from the 30 % fraction

Although the procedure to be described is applicable to any fraction of the extract containing receptor protein, use of the 30 % fraction markedly reduces the amount of curare needed. All steps are done at 0°. The 30 % fraction was thoroughly dialyzed against phosphate, pH 7.5, $\mu = 0.1$ (hereafter referred to as the phosphate buffer), followed by centrifugation at 20,000 rev./min to remove insoluble material. To the clear solution obtained, a concentrated solution of *d*-tubocurarine chloride (50–100 mg/ml) in distilled water was added until precipitation was complete. The approximate amount of curare required was determined before-hand on 1-ml aliquot of the protein solution. A small excess of curare was added to the bulk of the solution to insure completeness of precipitation. The suspension was kept at about 0° overnight and centrifuged at 4000 rev./min for about 30 min. The resulting precipitate was suspended in the phosphate buffer and dialyzed for several days against the same buffer, after which the suspension was centrifuged as before and the precipitate washed with the buffer. The precipitate was resuspended in Tris buffer, $\mu = 0.3$, pH 9, and dialyzed against this buffer until solubilization was complete (about 3 days). Dialysis was then continued against the phosphate buffer to adjust conditions to those used for binding studies or against 0.02 ionic strength buffer for lyophilization purposes.

Source of compounds used in binding studies

d-Tubocurarine dichloride obtained from K and K Chemical Co., was purified according to DUTCHER's procedure[13]. Chloride analysis revealed that the samples used were about 99 % pure. Dimethyl *d*-tubocurarine diiodide was a gift from Dr. O. K. BEHRENS of Eli Lilly Company. Its u.v. absorption spectrum and extinction coefficients were virtually identical with the same compound synthesized from our *d*-tubocurarine by the Tracerlab Co. of Boston, Mass. Prostigmine bromide was a gift from Dr. J. A. AESCHLIMANN, Hoffmann LaRoche. Atropine sulfate, eserine salicylate (Merck), methyl pyridinium iodide (K & K), succinylcholine dichloride (Winthrop-Stearns), and the various other choline derivatives were the commercially available compounds used. PAM and other pyridinium derivatives were synthesized by Dr. S. GINSBURG of this Laboratory.

Analytical procedures

Protein concentration was determined from u.v. O.D. using extinction coefficients which were based on dry weight. The u.v. absorption spectrum of the tissue extract is not that of a typical protein, *e.g.*, bovine serum albumin. As shown in Fig. 1 neither the 280 mμ maximum or 250 mμ minimum are exhibited by the extract; prolonged dialysis seemed to alter the spectrum somewhat. In dilute alkaline solution (pH 12) spectral changes were observed although once again different from those encountered

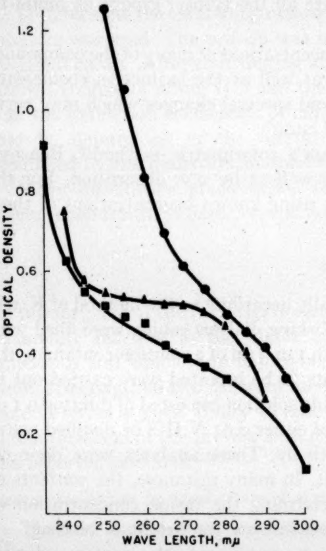

Fig. 1. U.v. absorption spectra of electric tissue extract, obtained from Electrophorus electricus. ■, undialyzed, diluent phosphate, pH 6.5; ▲, dialyzed, diluent phosphate, pH 6.5; ●, dialyzed, diluent 0.1 M NaOH.

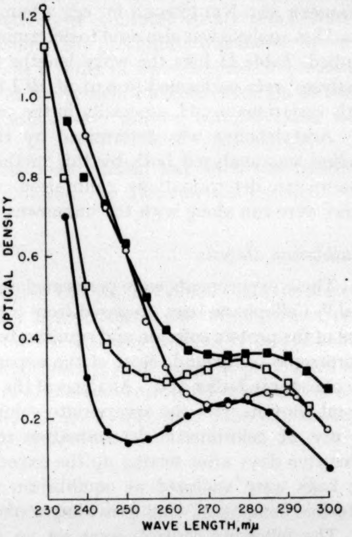

Fig. 2. U.v. spectra, 40% ammonium sulfate fraction and purified receptor. ●, 40% fraction, in 0.1 N HCl; □, receptor, 0.01 N HCl; ○, 40% fraction, in 0.1 N NaOH; ■, receptor, in 0.1 N NaOH.

TABLE II

U.V. ABSORPTION CHARACTERISTICS OF THE VARIOUS COMPOUNDS USED

Compound	*λ Max*	E_{mg}, *1 cm cell*
d-tubocurarine dichloride (curare)	280	11.21
Dimethyl *d*-tubocurarine diiodide (dimethyl curare)	280	8.0
Eserine salicylate	300	15.2
Prostigmine bromide	260	1.53
Atropine sulfate	260	0.43
Procaine hydrochloride	280	6.39
Pyridine 4 aldoxime methiodide	280	68.34
Pyridine 2 aldoxime dodeciodide	285	22.36
Hexamethylene bis-4 PAM	280	76.50
Decamethylene bis-4 PAM	280	70.14
Methyl pyridinium bromide	260	17.54
Cetyl pyridinium chloride	260	11.17
Hexamethylene bis-pyridinium	260	21.48
Decamethylene bis-pyridinium	260	19.94

with most proteins. However, the absorption spectrum of all the ammonium sulfate fractions as well as the purified receptor fraction were normal both at low and high pH (Fig. 2) although the shift occurring in alkaline solution was still not as pronounced as with bovine serum albumin. Whether this indicates a large proportion of H-bonded tyrosine residues, still only partially dissociated even at pH 12, has not been determined. A pH of 13 might be required to titrate all the tyrosyl groups as found by CRAMMER AND NEUBERGER for egg albumin[14].

U.v. analysis was also used to determine concentrations of many of the compounds studied. Table II lists the wave lengths used as well as the extinction coefficients. Analyses were performed in 0.01 M HCl to avoid spectral changes which may occur with variations in pH, especially in the case of curare.

Acetylcholine was determined by HESTRIN's colorimetric method[15]. Benzoylcholine was analyzed both by this method as well as by u.v. absorption. For the colorimetric determinations, calibration curves using known concentrations of these esters were run along with the unknowns.

Equilibrium dialysis

These experiments were performed essentially according to the method of KLOTZ *et al.*[16]. Cellophane bags prepared from 6 mm Visking dialysis tubing were filled with 1 ml of the protein solution and equilibrated with 1 or 3 ml of a solution containing the neurotropic compound. Most of the experiments to be reported were carried out in the phosphate buffer at 0°. Analysis of the outside solution consisted of diluting 0.1 or 0.2-ml aliquots with the appropriate volumes of either 0.01 N HCl or distilled water for u.v. or colorimetric determination respectively. These analyses were done on successive days after setting up the experiment. In many instances, the contents of the bags were analyzed at equilibrium to determine the inside concentration of diffusible compound, thus providing a direct measure for the extent of binding.

The following controls were set up for each experiment: the protein solution dialyzed against buffer, solution of neurotropic compound against buffer and buffer against buffer. The first control was necessary since u.v. absorbing and ninhydrin reacting material frequently appeared in these dialyzates. From control 2 it was established that no significant binding of the various compounds to the dialysis bag had occurred and that at equilibrium the concentration of diffusible compound inside and outside of the bag were identical within experimental error. On the other hand, it could be shown that in some instances, *i.e.*, at concentrations above 10^{-3} M the formation of micellar aggregates evidentally prevented equilibration across the membrane of both CPC and PAD. Control 3 was necessary to correct for the small but significant amount of u.v. absorbing material elaborated from the bags themselves.

Calculation of the amount of binding was based on the outside concentration of the compound when dialyzed against the protein as compared with the concentration in the buffer control. These values were in good agreement with those obtained from analyses of the inside solutions of the protein set ups at equilibrium.

Experiments involving the various choline derivatives were performed in the presence of 20 μg of TEPP in order to eliminate acetylcholinesterase activity. Although the amounts of enzyme are extremely small in absolute values, enough is present to interfere with experiments carried out over a period of many hours. Analysis by titration at constant pH after treatment with TEPP revealed the complete absence of enzymic activity at the beginning of the experiment. However, about 5 days later a small fraction of the activity had returned. It is assumed that during the first 3 days of the experiment no activity was present since the concentrations of the choline compounds in dialyzates on two successive days remained constant.

Precipitation studies

To determine the combining ratio of curare with the receptor protein the following procedure was used: The protein was exhaustively precipitated with a concentrated solution of curare. After centrifuging and washing, the precipitate was dissolved with a known volume of dilute HCl and the O.D. at a series of wave lengths determined. From the extinction coefficients of curare and the protein the concentration of both could be determined in the mixture. Alternatively, protein concentration was determined by the ninhydrin method[17].

Many qualitative precipitation reactions were carried out with the receptor protein, chondroitin sulfate, bovine serum albumin, egg albumin and casein.

RESULTS

The primary aim of the investigations reported in this paper was to isolate the protein presumably reacting in the intact cell with acetylcholine and other neurotropic compounds and which may be responsible for the biological effects of acetylcholine action. This required two different lines of experiments: (a) testing the proteins of the tissue in terms of their ability to bind curare and other neurotropic compounds and (b) identification of the protein as the receptor. A few preliminary observations have been reported previously[18, 19].

Curare interaction by equilibrium dialysis

The first studies using unfractionated tissue extract revealed that there was no

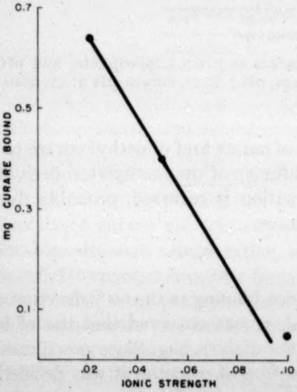

Fig. 3. Binding of curare to 60% fraction as a function of ionic strength at pH 7.5. Initial curare concn., 0.3 mg/ml. Phosphate, 0.02 μ with NaCl contributing the remaining ionic strength.

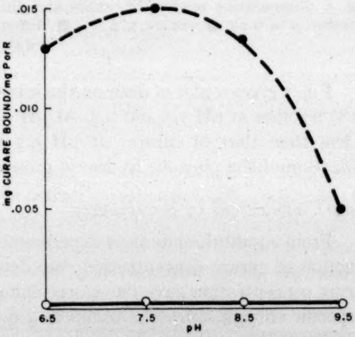

Fig. 4. ●, binding of curare to 60% fraction; ○, to tissue residue, as a function of pH at ionic strength 0.1. Initial curare concn., 0.3 mg/ml. Ordinate: mg curare bound/mg protein (P) or tissue residue (R).

significant binding of curare at an ionic strength of 0.1. This finding may be explained by the fact that the concentration of receptor present in the intact extract is too small to provide for measurable interaction. Complexing to the 60 % fraction at 0.1 ionic strength could be readily demonstrated (Fig. 3), binding being greatly enhanced by decreasing ionic strength. Fig. 4 shows that binding to the 60 % fraction is optimal at pH 7.5 as well as the fact that binding to the residue remaining after extraction was so negligible that this material was not used for further study.

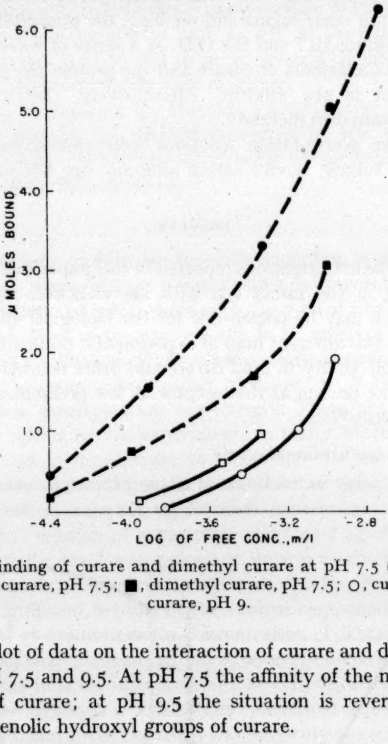

Fig. 5. Comparative binding of curare and dimethyl curare at pH 7.5 (phosphate) and pH 9.5 (borate); $\mu = 0.02$. ●, curare, pH 7.5; ■, dimethyl curare, pH 7.5; ○, curare, pH 9; □, dimethyl curare, pH 9.

Fig. 5 gives a plot of data on the interaction of curare and dimethyl curare to the 60 % fraction at pH 7.5 and 9.5. At pH 7.5 the affinity of the methylated derivative is less than that of curare; at pH 9.5 the situation is reversed, probably due to ionization of the phenolic hydroxyl groups of curare.

Curare interactions by precipitation

From equilibrium dialysis experiments in which binding to the 60 % fraction as a function of curare concentration was determined, it was observed that use of high curare concentration gave rise to precipitates in the dialysis bag. Since precipitation, if specific enough, might be useful as a quick measure of receptor, it was decided to investigate this property of curare more extensively. In extending these studies it was found that curare not only precipitates material in the extract, but RNA, DNA and chondroitin sulfate A as well—the latter reaction having been studied rather extensively and reported elsewhere[8–10]. Precipitation of both chondroitin sulfate and the component of the 60% fraction had the features in common that, at fairly low concentration of macromolecule, precipitation was surpressed by increasing μ (Table III). This was in complete agreement with the equilibrium dialysis data. However, the character of the precipitates once formed was found to be radically different insofar as lability to salt was concerned. Thus while the curare—chondroitin sulfate precipitate was readily soluble in dilute salt and could be rapidly solubilized by dialysis against phosphate buffer at an ionic strength of 0.1, the tissue component—curare precipitate was quite stable to increases in ionic strength and failed to dissolve to any significant degree despite prolonged dialysis against the phosphate buffer. Both types of precipitate were readily soluble in dilute acid and base as well as in 1–2 *M* urea (Table IV), the latter suggesting that precipitation involves H-bonds. In accordance with these observations, dialysis of the curare—tissue component precipitate against buffer at pH 9, at which pH the phenolic hydroxyls of curare are ionized, resulted in complete solublization. Tris buffer is preferable, although solubilization in borate buffer at the same pH could also be achieved. However, it was later found that borate actually potentiated the interaction of curare with some proteins. Thus bovine serum albumin which ordinarily reacted weakly with curare (see below), precipitated with that substance in the presence of borate. The procedure used for isolating the receptor protein in solution depended on the lability of the precipitate in high pH buffer as previously described.

TABLE III

EFFECT OF IONIC STRENGTH ON PRECIPITATION OF COMPONENT OF TISSUE EXTRACT AND CHONDROITIN SULFATE A BY CURARE

Final curare concn., 5 mg/ml, pH 7.5.

Ionic strength	*Unfractionated tissue extract*	*60% fraction*	*Chondroitin sulfate*
0.006	Dense precipitation	Precipitation	Precipitation
0.11	Precipitation	Turbid	Slight precipitation
0.16	Slight precipitation	Clear	Slight turbidity
0.20	Slight precipitation	Clear	Clear
0.26	Clear	Clear	Clear

TABLE IV

EFFECT OF UREA ON CURARE PRECIPITATION

Urea Concn., M	*60% fraction*	*Chondroitin sulfate A*
0	Precipitation	Precipitation
1.0	Precipitation	Turbid
2.0	Turbid	Clear
3.0	Clear	Clear
4.0	Clear	Clear

It is to be noted that curare precipitation of the receptor protein occurred at much lower concentrations of both reactants than with other extract fractions, indicating a high degree of purification (Table V).

At room temperature, curare at very high concentrations failed to precipitate egg albumin, bovine serum albumin, pepsin, trypsin and lysozyme. Precipitation with isoelectric, unfractionated casein, as well as with the α and β fractions of that protein* were observed and will be discussed below. At 60°, curare caused a rapid rise in the viscosity of bovine serum albumin and subsequent precipitation of the protein.

As an indication of the usefulness of curare precipitation, the data of Table VI may be cited. This experiment shows quite clearly that the major portion of the curare precipitating component, later identified as the receptor protein, is present in the 30 % ammonium sulfate fraction. By contrast, the long chain quaternary CPC, which also reacts with the receptor, yielded precipitates in all fractions tested. Evidentally, precipitation by this compound is far too non-specific to be of use either in localizing or in isolating the receptor.

Precipitation studies using other quaternary compounds

Table VII summarizes the results of a great number of precipitation studies involving a large number of mono- and bis-quaternary compounds, all of which interact with the receptor as shown by studies on intact cells. These compounds seem to fall into three distinct types insofar as their precipitating ability is concerned: (a) Non-precipitating quaternary and bis-quaternary compounds. (b) Bis-quaternary compounds with a relatively high degree of specificity, *i.e.*, they do not precipitate egg albumin. These include curare, dimethyl curare, stilbamidine and decamethylene bis-PAM. (c) Mono-quaternary compounds which precipitate with a lesser degree of specificity. Examples include DPC, PAD, and nor-acetylcholine 12.

Binding studies with other compounds

All of these studies were carried out at pH 7.5, $\mu = 0.1$, using various ammonium sulfate fractions as well as the receptor protein. Results using several series of compounds are shown in Figs. 6–8.

DISCUSSION

The results presented in this paper show that it has been possible to isolate and to identify the acetylcholine receptor.

Several criteria for identification of the receptor have been used in the present investigations. The molecule must first of all have the ability to bind acetylcholine analogues and related compounds in a graded fashion consistent with that demonstrated by their affinity for the receptor in the intact electroplax. Based on studies in solution, it should be possible to make predictions about the effectiveness of previously untested compounds on the electrical activity of the electroplax. Effects of pH, ionic strength, specific ions, temperature etc., on binding in solution should yield results which agree at least in a qualitative way with observations on the living cell. The material must be obtained in quantities compatible with the information available about the amount in the cell.

Binding of various compounds in solution and the relation to their effects on electrical activity

The affinity of the various compounds to the proteins of the 30 % and 60 % ammonium sulfate fractions as well as to the curare precipitating protein has been

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* Generously provided by Dr. G. PERLMANN of the Rockefeller Institute, New York City.

TABLE V

SENSITIVITY OF VARIOUS PREPARATIONS TO CURARE PRECIPITATION

pH 7.5, $\mu = 0.1$.

Preparation	*Protein concn. mg/ml*	*Curare concn. for first ppt. mg/ml*
Tissue extract	25	10.5
60 % fraction	23	6
30 % fraction	4	4.5
Receptor	0.08	2

TABLE VI

PRECIPITATION OF VARIOUS AMMONIUM SULFATE FRACTIONS BY CURARE AND BY CPC

$(NH_4)_2SO_4$ fraction	*Curare*	*CPC*
0–30 % saturation	++++	++++
30–40 % saturation	+	++++
40–50 % saturation	—	++++
50–60 % saturation	—	++++

TABLE VII

QUALITATIVE PRECIPITATION REACTIONS BETWEEN VARIOUS COMPOUNDS AND A NUMBER OF MACROMOLECULES

	Receptor protein	*Unfractionated Casein*	*BSA*	*Egg albumin*	*Chondroitin sulfate*
Curare	++++	++++	— (++ at 60°)	—	++++
Dimethyl curare	+	+	—	—	+
Prostigmine	Negative throughout				
Eserine	Negative throughout				
Atropine	Negative throughout				
Decamethonium	Negative throughout				
Acetylcholine	Negative throughout				
Carbamylcholine	Negative throughout				
Benzoylcholine	Negative throughout				
Butyrylcholine	Negative throughout				
Noracetylcholine 12	+++	+++	—	+++	+++
Norcholine 12	+++	+++	—	+++	+++
Tetrabutyl ammonium	—	—	—	—	—
Methyl pyridinium	—	—	—		—
Dodecyl pyridinium	+++	+++	—	++	+++
Cetyl pyridinium	++++	++++	—	+++	++++
Hexamethylene bis-pyridinium	—	—	—		—
Decamethylene bis-pyridinium	±	±	—		—
Hexamethylene bis-4-PAM	±	++	— (— at 60°)		—
Decamethylene bis-4-PAM	++	++	— (+ at 60°)		—
Stilbamidine	++	++	— (++ at 60°)		++

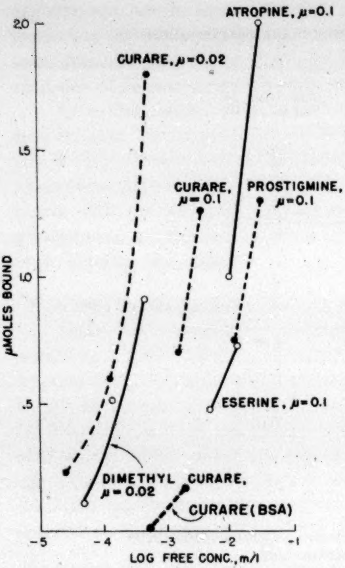

Fig. 6.

Fig. 6. Binding of curare and related nitrogen compounds to 60% fraction, pH 7.5, $\mu = 0.1$ (phosphate). Protein concn., 15 mg/ml. The various compounds are indicated in the figure. Binding of curare and dimethyl curare at $\mu = 0.02$ is shown for comparison. Also shown is the binding of curare by BSA, 10 mg/ml, under similar conditions.

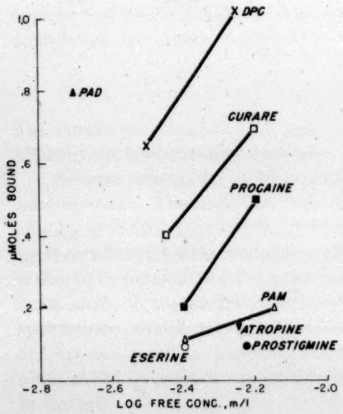

Fig. 7.

Fig. 7. Binding of curare and related compounds to 30% fraction, pH 7.5, $\mu = 0.1$ (phosphate) protein concn., 4.5 mg/ml.

Fig. 8. ●, binding of acetylcholine, ○, binding of benzoylcholine, to purified receptor. A single point (■) is shown for acetylcholine interaction with BSA.

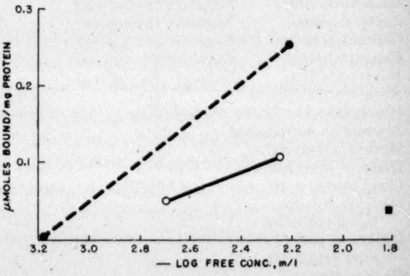

Fig. 8.

shown to follow in a qualitative way the ability of these compounds to interfere with the electrical activity of the intact cell. Thus the order of binding is: curare, procaine, atropine, eserine and Prostigmine. Dimethyl curare has been found in the present studies to be more weakly bound in solution than curare itself; this seemed to be in contradiction to results repeatedly reported from whole animal experiments[13, 21]. However, when tested on the intact electroplax, the methylated derivative indeed proved to be a weaker blocking agent than curare[22]. The stronger binding of curare to the receptor may be explained by the two phenolic hydroxy groups which contribute to the binding force by the formation of hydrogen bonds, a fact which was demonstrated in solution. The difference in behavior of these two compounds in the whole animal and on the single cell must be attributed to factors other than affinity to receptor. A possible explanation is offered by the finding that dimethyl curare is slightly chloroform soluble, apparently enabling it to penetrate to regions inaccessible to curare, a factor which may be important in effects on the animal.

Other comparisons among quaternary compounds of a given series revealed a parallelism insofar as binding in solution and effect on electrical activity were concerned. In both types of studies, PAD was superior to PAM, dodecyl pyridinium better than methyl pyridinium. Perhaps most striking was the finding that the order of affinities: decamethylene bis-PAM > hexamethylene bis-PAM > PAM and decamethylene bis-pyridinium > hexamethylene bis-pyridinium > methyl pyridinium as determined in solution (Fig. 9) was completely maintained in experiments of ROSENBERG AND HIGMAN on the electroplax[22]. The bis compounds of these series had not previously been tested on the cell. Another aspect of these findings will be discussed below.

As for the choline compounds, only the binding of acetylcholine and benzoylcholine to the receptor has been examined thusfar. Although the data are only preliminary, the order of binding in solution and effect on the cell yielded parallel results at least insofar as demonstrating the superiority of acetylcholine.

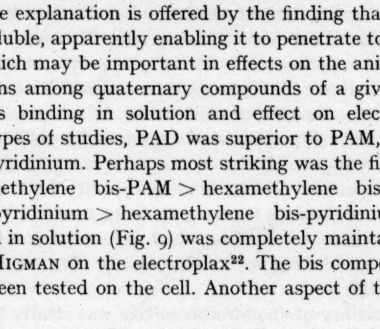

Fig. 9. Binding of bis-quaternary ammonium compounds to the receptor protein pH 7.5, $\mu = 0.1$ (phosphate). Protein concn., 10 mg/ml. Abbreviations: HBPB, hexamethylene bis-pyridinium; DBPB, decamethylene bis-pyridinium; H bis PAM, hexamethylene bis-PAM; D bis PAM: decamethylene bis-PAM.

Specificity of binding in comparison with other macromolecules

On the basis of studies using other macromolecules, it is apparent that binding in a graded fashion is by itself not a sufficiently specific criterion to identify a molecule as the receptor. For example, considering unpublished data on the binding to chondroitin sulfate, it might be concluded that this substance also exhibits receptor-like activity inasmuch as the gradation in affinities of a number of compounds also may in some instances parallel that of the *in vivo* studies. Moreover, curare quantitively precipitates this polysaccharide and does so in a most interesting fashion: the curare molecule seems to have the exact dimensions to fit between the two negative charges of the chondroitin sulfate monomer[8-10]. It would therefore appear that a molecule of this sort possesses some of the structural requirements to act as a receptor for curare and other curare-like agents, most of which are di-quaternary compounds presumably having a 14–15 Å distance between cationic groups. However, on the basis of at least four different types of experimental evidence, this polysaccharide may be eliminated as the physiological receptor: (a) Although chondroitin sulfate does bind acetylcholine and other choline esters, the affinities in solution do not parallel the effectiveness of these compounds on the cell (unpublished experiments). (b) The configuration of the molecule is such that it apparently does not interact in a graded fashion with a variety of di-quaternary compounds. (c) The binding capacity for curare and related compounds on a mole to mole basis is far too great to be reconciled with WASER's data on binding to the intact tissue. (d) Binding which does occur is more or less non-specific *i.e.*, not relegated to specific regions in the molecule. The latter three considerations will be expanded on below.

The lack of versatility of chondroitin sulfate was clearly brought out when the interaction of methyl pyridinium was compared with that of hexamethylene and decamethylene bis-pyridinium or the corresponding PAM series. It was found that the polysaccharide could hardly distinguish the various bis-quaternary compounds from the mono-quaternary[9]. The binding of all the derivatives was almost identical and very weak. On the other hand, (Fig. 9) the receptor protein showed a distinct gradation in affinity towards these various compounds, in complete agreement with their effects on the intact cell.

While the distance between adjacent negative charges in the condroitin sulfate molecule appears to be suitable for accommodating two cationic groups in a given molecule, compounds with a rigid structure apparently combine more readily with two such sites. Thus of the many di-quaternary compounds examined (Table VI) only curare and stilbamidine precipitate chondroitin sulfate. Non-rigid molecules such as decamethonium or the decamethylene derivatives discussed apparently are not readily bound simultaneously to the two adjacent negative groups. Thus these compounds interact essentially like the mono-quaternary compounds. Conversely, a protein molecule could have many contributory side chains or secondary foldings which become involved in binding and in this way show a gradation in affinity for the great variety of compounds or neurotropic agents known to be bound to the receptor. This is particularly true insofar as curariform action is concerned: although the optimum distance between the two cationic nitrogens is 14–15 Å, pronounced effects may be elicited with molecules differing greatly with respect to this distance[23]. This fact alone suggests that the receptor may have some configurational adaptability, perhaps similar to that proposed by KARUSH for the bovine serum albumin molecule[24,25], a property evidentally absent in polysaccharides and nucleic acids. Alternatively, the receptor may also impose alterations in structure of the small molecule.

The other point of interest concerns the comparative curare-binding capacity of the curare-precipitating protein and chondroitin sulfate, *i.e.*, the number of curare molecules bound at saturation. It may be expected, by analogy with enzymes, that the receptor may have a few binding sites and that perhaps the binding of one or two curare molecules would be sufficient to produce the physiological effects of the drug. From Fig. 10 it can be seen that, at saturation, approximately 0.1 mg curare is bound/mg receptor protein. This value is in good agreement with that obtained by precipitating the receptor and analyzing the precipitate for both components. This corresponds to about 10 curare molecules/molecule of protein, assuming a molecular weight of 100,000. On the other hand, chondroitin sulfate has a much higher combining capacity for curare[8,9], corresponding to about 150 curare molecules bound/100,000 molecular weight, or a complete neutralization of all the charges on the polysaccharide. This order of magnitude of binding is inconsistent with the results of WASER[20] who found by auto-radiographic techniques on intact rat diaphragm that a single receptor molecule most likely will combine with but a few curare molecules at saturation.

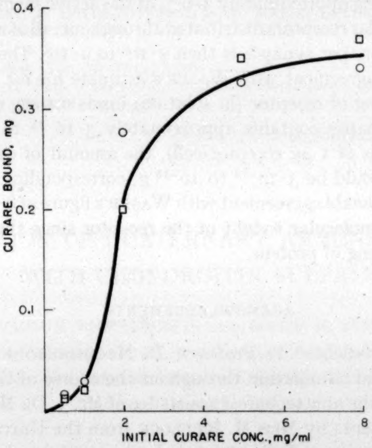

Fig. 10 Binding of curare to purified receptor protein prepared from 30% fraction □, and from tissue extract ○. pH 7.5 $\mu = 0.1$ (phosphate). Protein concn. 3 mg/ml.

Effect of other variables on curare binding in solution and on its action on the intact cell

Reduction of ionic strength in terms of Na ion concentration greatly enhances the affinity of curare to its cellular receptor as well as to the tissue component in the extract. Urea may be employed for dissociating the complex in the test tube, presumably because of its hydrogen bond breaking ability. The effect of urea on curare action in the intact cell is under study, but it may be noted that guanidine, another compound known to break hydrogen bonds, has been reported to be antagonistic to curare[26, 27].

Calcium is also effective in dissociating the curare–protein complex. Increasing the Ca concentration from 2 to 8 m*M* resulted in the abolition of the action of curare (2 μg/ml) on the intact electroplax The effect of Ca suggests that the curare binding site might contain one or more phosphate groups and indeed the receptor protein has been found to contain a significant amount of phosphate. Curare precipitates α-casein more readily than β-casein; this observation also suggests that curare interaction with proteins may involve phosphate groups whereas carboxyls may play only a limited role. Proteins such as serum albumin and egg albumin, having many carboxyl groups but devoid of phosphate, failed to precipitate with curare (Table VI).

Binding of curare by the receptor protein in relation to the amount fixed to tissue

A rough estimate may be made of the number of receptors per cell as well as the number of curare molecules bound per synapse at saturation. From the weight of tissue used the approximate number of electroplax extracted may be estimated. The amount of receptor obtainable from the extract by curare precipitation has been determined on a number of occasions. The receptor per cell has been estimated to be about 1 μg. Assuming a molecular weight of 100,000, this gives a total of 10^{-11} moles receptor or $6 \cdot 10^{12}$ molecules/cell. There are approximately 3–$10 \cdot 10^{4}$ synapses per electroplax[4], constituting approximately 3–6 % of the active membrane which may be considered to have similar receptors distributed throughout, all of which are extractible. The number of receptors per synapse is then $2 \cdot 10^{6}$ to $2 \cdot 10^{7}$. These admittedly crude estimates are in good agreement with WASER's estimate for rat diaphragm[20].

At saturation, 1 mg of receptor (in solution) binds 0.1 mg curare (approx. 10^{-7} moles). Since each synapse contains approximately $3 \cdot 10^{-11}$ to 10^{-12} g of receptor (calculated on the basis of 1 μg receptor/cell), the amount of curare expected to be bound at saturation would be $3 \cdot 10^{-14}$ to 10^{-13} g, corresponding to between 2.4 and $7 \cdot 10^{7}$ molecules, in reasonable agreement with WASER's figure of $8 \cdot 10^{6}$. This calculation is independent of the molecular weight of the receptor since the combining ratio in solution is in terms of mg of protein.

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REFERENCES

[1] D. NACHMANSOHN, *Chemical and Molecular Basis of Nerve Activity*, Academic Press, New York, 1959.

[2] D. NACHMANSOHN AND I. B. WILSON, *Advances in Enzymology*, 12 (1951) 259.

[3] M. ALTAMIRANO, W. L. SCHLEYER, C. W. COATES AND D. NACHMANSOHN, *Biochim. Biophys. Acta*, 16 (1955) 268.

[4] E. SCHOFFENIELS AND D. NACHMANSOHN, *Biochim. Biophys. Acta*, 26 (1957) 1.

[5] I. B. WILSON AND E. CALIB, *J. Biol. Chem.*, 78 (1956) 202.

[6] C. CHAGAS, E. PENNA-FRANCA, A. HASSON, C. CROCKER, K. NISHIE AND E. J. GARCIA, *Ann. acad. brasil. sci.*, 29 (1957) 53.

[7] C. Chagas, E. Penna-Franca, K. Nishie and E. J. Garcia, *Arch. Biochem. Biophys.*, 75 (1958) 251.
[8] S. Ehrenpreis and M. M. Fishman, *Abstracts 135th Meeting Am. Chem. Soc., Boston, April 1959*, p. 7D.
[9] S. Ehrenpreis and M. M. Fishman, *Biochim. Biophys. Acta*, 44 (1960) 577.
[10] S. Ehrenpreis, in preparation.
[11] C. Chagas, *Ann. N.Y. Acad. Sci.*, 81 (1959) 345.
[12] M. A. Rothenberg and D. Nachmansohn, *J. Biol. Chem.*, 168 (1947) 223.
[13] J. D. Dutcher, *J. Am. Chem. Soc.*, 68 (1946) 419.
[14] J. L. Crammer and A. Neuberger, *Biochem. J.*, 37 (1943) 302.
[15] S. Hestrin, *J. Biol. Chem.*, 180 (1949) 249.
[16] I. M. Klotz, F. M. Walker and R. B. Pivan, *J. Am. Chem. Soc.*, 68 (1946) 1486.
[17] V. J. Harding and R. M. MacLean, *J. Biol. Chem.*, 24 (1916) 503.
[18] S. Ehrenpreis, *Science*, 129 (1959) 1613.
[19] S. Ehrenpreis, *Federation Proc.*, 18 (1959) 220.
[20] P. Waser, in D. Bovet, F. Bovet-Nitti and G. B. Marini-Bettolo, *Curare and Curare-like Agents*, Elsevier Publishing Co., 1959, p. 219.
[21] O. Wintersteiner and J. D. Dutcher, *Science*, 97 (1943) 467.
[22] P. Rosenberg and H. Higman, *Biochim. Biophys. Acta*, to be submitted.
[23] D. Bovet, in D. Bovet, F. Bovet-Nitti and G. B. Marini-Bettolo, *Curare and Curare-like Agents*, Elsevier Publishing Co., 1959, p. 219.
[24] F. Karush, *J. Am. Chem. Soc.*, 72 (1950) 2705.
[25] F. Karush, *J. Am. Chem. Soc.*, 76 (1954) 5536.
[26] H. Fuhner, *Arch. exptl. Path. Pharmakol. Naunyn-Schmiedeberg's*, 58 (1908) 1.
[27] T. P. Feng, *Chinese J. Physiol.*, 13 (1938) 119.

THE INTERACTION OF QUATERNARY AMMONIUM COMPOUNDS WITH CHONDROITIN SULFATE

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SUMMARY

The interaction of a series of mono- and di-quaternary ammonium compounds with chondroitin sulfate A has been studied. Quantitative precipitation of the polysaccharide occurs with cetyl pyridinium chloride and *d*-tubocurarine. The ratio, moles quaternary bound/mole repeating unit of polysaccharide, is 2 for CPC and 1 for curare. Evidence is presented to show that the rigid curare molecule precipitates by combining simultaneously with both the carboxyl and sulfate groups of the repeating unit of ChSA, rather than causing inter-molecular aggregation. Di-quaternary compounds which do not have a rigid structure fail to precipitate chondroitin sulfate and show only weak interactions in solution.

Abbreviations: CPC, cetyl pyridinium chloride; MPB, methylpyridinium bromide; ChSA, chondroitin sulfate A; DPC, dodecyl pyridinium chloride; MPB, methyl pyridinium bromide; PAM, pyridine 4 aldoxime methiodide; HBPB, hexamethylene bis-pyridinium; DBPB, decamethylene bis-pyridinium.

INTRODUCTION

Numerous investigations have been carried out on the interaction of long-chain mono-quaternary ammonium compounds with both acidic and neutral polysaccharides[1–4]. As a result of such studies, methods for the isolation of polysaccharides from various tissues have been developed, based on precipitation with these quaternary compounds[5–7]. On the other hand, interactions with di-quaternary ammonium compounds have, in general, been neglected. Such reactions may be of particular interest for at least two reasons: (a) The repeating units of many acidic polysaccharides are bi-functionally charged; thus, studies of this kind may provide information about the configuration of these macromolecules in solution which in turn may lead to an understanding of how such polysaccharides are built into ground substance of connective tissue. (b) Diquaternary ammonium derivatives have powerful pharmacological action and find increasing use in medicine. One such compound, the nerve poison *d*-tubocurarine (curare) has recently been used for the selective precipitation and isolation of the specific acetylcholine receptor protein[8,9]. Since curare also forms a precipitate with chondroitin sulfate A (see ref. 10), it would be of some interest to evaluate the possible role which acidic polysaccharides play in cellular reactions of curare and other bis-quaternary compounds. This is particularly pertinent since CHAGAS has attributed a function to acidic polysaccharides in terms of binding quaternary ammonium ions, and has ascribed receptor activity to some polysaccharides[11,12].

MATERIALS AND METHODS

Quaternary ammonium compounds

The quaternary compounds used, together with some of their u.v. absorption characteristics, are given in Table I. Structures of most of these compounds are shown in Fig. 1. The compounds were dissolved in phosphate buffer, pH 7.5, $\mu = 0.02$, immediately before use. This is particularly important with regard to curare which turns yellow on standing even at 0°. The final concentration of the compounds used in the various reactions was such that their contribution to the ionic strength did not appear to affect the results.

TABLE I

U.V. ABSORPTION CHARACTERISTICS OF VARIOUS QUATERNARY AMMONIUM COMPOUNDS

Compound	*λ max*	*Emg, 1 cm cell*
d-tubocurarine dichloride (curare)	280	11.4
Dimethyl *d*-tubocurarine diiodide (dimethyl curare)	280	8.0
Cetyl pyridinium chloride	260	11.2
Dodecyl pyridinium chloride	260	14.7
Methyl pyridinium bromide	260	20.0
Hexamethylene bis-pyridinium	260	21.5
Decamethylene bis-pyridinium	260	19.9
Pyridine 4 aldoxime methiodide	280	68.3
Hexamethylene bis-4 PAM	280	76.5
Hexamethylene-4-PAM	280	65.0
Decamethylene bis-4 PAM	280	70.1
Na chondroitin sulfate	260	0.3
	280	0.25

The CPC, MPB and curare were obtained from the K and K Chemical Company of New York. The dimethyl curare was a gift from Dr. O. K. BEHRENS of Eli Lilly and Co.

The other compounds were synthesized by Dr. S. GINSBERG of this laboratory. All compounds were used without further purification.

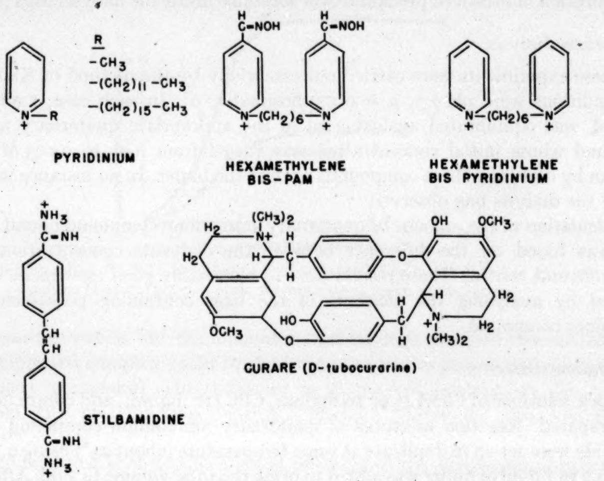
R
$-CH_3$
$-(CH_2)_{11}-CH_3$
$-(CH_2)_{15}-CH_3$
PYRIDINIUM
HEXAMETHYLENE BIS-PAM
HEXAMETHYLENE BIS PYRIDINIUM
CURARE (D-tubocurarine)
STILBAMIDINE

Fig. 1. Chemical structure of some of the quaternary ammonium ions used in this study.

Chondroitin sulfate A

The ChSA used in this investigation was obtained from various commercial sources and was used either as received or was purified following the method of EINBINDER AND SCHUBERT[13]. Prior to use, the polysaccharide was dissolved in the phosphate buffer at pH 7.5, $\mu = 0.02$, and dialyzed overnight at 0°.

A highly purified sample of ChSA was also supplied by Prof. K. MEYER, Department of Medicine, Columbia University. The uronic acid content of our purified preparations compared favorably with this sample, both by orcinol and carbazole analyses. On this basis, these ChSA preparations were about 90 % pure.

The structure of the ChSA repeating unit[14] is shown in Fig. 2.

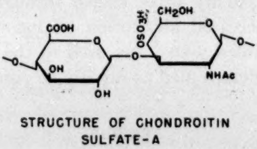
STRUCTURE OF CHONDROITIN SULFATE-A

Fig. 2. Chemical structure of the repeating unit of chondroitin sulfate A.

Spectrophotometry

All reaction mixtures were analyzed with a model DU Beckman spectrophotometer. This method was particularly applicable to these experiments inasmuch as the quaternary compounds have relatively high u.v. absorption spectra whereas ChSA shows only negligible absorption under these conditions (see Table I). Thus it was possible to check many of the results based on indirect measurements of precipitation and equilibrium dialysis experiments by direct analysis, *i.e.*, by determining the absorption of dissolved precipitates or solutions inside the dialysis bags (see below).

Equilibrium dialysis

These experiments were carried out essentially by the method of KLOTZ *et al.*[15]. The conditions were pH 7.5, $\mu = 0.1$ (phosphate), 0°. In each case, 1 ml of ChSA, 4 mg/ml, was equilibrated against 3 ml of the appropriate quaternary ammonium compound whose initial concentration was varied from 0.01 to 0.003 *M*. Controls were run by dialyzing these compounds against the buffer. In no instance was adsorption by the dialysis bag observed.

Calculation of the amount of quaternary ammonium compound bound at equilibrium was based on the difference between the dialyzate concentrations of polysaccharide and control. These results were in reasonably good agreement with those obtained by analyzing the contents of the bags containing polysaccharide and ammonium compound.

Precipitation studies

Stock solutions of ChSA (5 or 10 mg/ml), CPC (10 mg/ml), and curare (20 mg/ml), were prepared. Reaction mixtures of quaternary ammonium compound and polysaccharide were set up in duplicate at room temperature (about 23°) using 0.1 or 0.2 ml ChSA. 0.2 to 1.8 ml of buffer was added to bring the total volume to 2 ml. After precipitation had occurred, the solutions were allowed to stand for at least 15 min before centrifuging at 4000 rev./min, also for 15 min. Aliquots of the supernatants were removed for analysis as described above; u.v. absorption was used for the quaternary ammonium compound, orcinol for the ChSA.

It had been observed that some mixtures gave turbid supernatants, especially at the phase of precipitation prior to the equivalence point (see below). In order to avoid turbidities in the solutions read with the spectrophotometer, dilutions of supernatants were made with 3 *M* NaCl for the CPC analysis and 1 *M* NaCl for curare. During the course of the initial studies it was found that passage of u.v. light at 260 mμ through the NaCl solution gave rise to a progressive appearance of some substance having a distinct u.v. absorption maximum at this same wave length. Recrystallization of the NaCl or use of doubly-distilled water failed to suppress this effect. Passage of light of 280 mμ wave length through the same solution had no effect on the NaCl. The increased absorption at 260 mμ interfered with the CPC analysis and therefore distilled water was used in the reference cuvette, the O.D. of the CPC solutions being determined as rapidly as possible.

RESULTS

The precipitation of ChSA by both CPC and curare is shown in Fig. 3. μmoles of the quaternary compounds bound are plotted as a function of their initial concentration. The following phases of the reaction may be distinguished: (a) A phase in which soluble complexes have been formed but no precipitation has taken place; this was demonstrated by equilibrium dialysis. (b) There is precipitation as well as a turbid supernatant but equivalence has not been reached. (c) An apparent equivalence in which the supernatant is clear but precipitation is nervertheless incomplete. (d) A true equivalence point. (e) A post-equivalence phase in which the quaternary compound is now present in excess but the amount bound remains constant. No solubilization of the precipitated complex could be detected even in the presence of a large excess of quaternary compound. This behavior is in marked contrast to the situation encountered with proteins where excess long-chain quaternary may solubilize the precipitate[16].

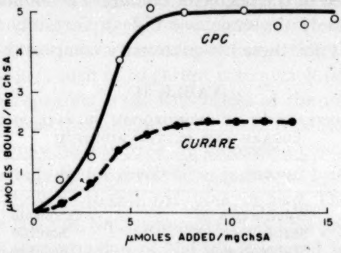

Fig. 3. Combining ratios of CPC and curare to chondroitin sulfate A, expressed as μmoles bound/mg polysaccharide. pH 7.5, ionic strength 0.02, room temperature.

It is to be noted that no ChSA could be found in the supernatants either at the equivalence point or in the post-equivalence zone, indicating complete precipitation of the polysaccharide material.

Perhaps the most interesting feature of these precipitation reactions concerns the molar ratios of CPC and curare to ChSA, bound at the equivalence point and in the post-equivalence phase. It is evident (Fig. 3) that the ratio of CPC to chondroitin sulfate monomer is approximately 2:1 at equivalence, whereas that of curare is 1:1. In other words, at equivalence, the number of moles of CPC bound is twice that of curare. These ratios held regardless of the concentration of ChSA, which was varied from 1 to 4 mg/ml. Moreover, this value was independent of the purity of ChSA used. Data from solubilized precipitates were in good agreement with those obtained from analysis of the supernatant. It is also to be noted that the maximum binding of curare, as determined by equilibrium dialysis, was in excellent agreement with that determined by precipitation. Equilibrium dialysis could not be run with CPC above about 10^{-3} *M* due to micelle formation which prevented equilibration across the dialysis bag.

Additional differences in the behavior of the two types of complexes studied were indicated by observations on the properties of the precipitates (Table II). It is evident that the CPC–ChSA precipitate is quite stable to low concentration of salt, to heat, urea, acid, and alkali. In contradistinction, the curare complex is labile to each of these agents. A distinct difference in solubility in organic solvents is also to be noted. The CPC precipitate was soluble in 95% alcohol as well as in acetone, whereas the curare precipitate was almost insoluble in both of these solvents.

Equilibrium dialysis data on the binding of various other pyridinium compounds are shown in Fig. 4. It is evident that the order of binding is CPC > DPC > MPB. In this range of concentration, the binding of CPC is stronger than curare.

The binding of bis-quaternary compounds of two series—hexamethylene-bis-PAM, decamethylene-bis-PAM and the corresponding bis-pyridiniums (see Table I)—was compared with that of the corresponding methylated mono-quaternary-PAM and MPB. Initial molar concentrations of each compound were identical in order to provide direct comparison of the degree of binding. The affinity of all six compounds to ChSA was approximately the same (see Fig. 5). Certainly the polysaccharide has a markedly lower affinity for these bis-quaternary compounds than for curare.

TABLE II

SOLUBILITY CHARACTERISTICS OF CHONDROITIN SULFATE PRECIPITATES WITH CURARE AND CETYLPYRIDINIUM

	CPC	*Curare*
NaCl	Soluble in 1 *M*	Soluble in 0.1 *M*
Temperature (70°)	Stable	Soluble
Urea	Stable in 6 *M*	Soluble in 1 *M*
pH	Stable pH 3.5–9.5	(a) Slightly soluble, pH 3.5 (b) Completely soluble, pH 2 (c) Completely soluble, pH 12
Ethyl alcohol, acetone	Soluble	Insoluble

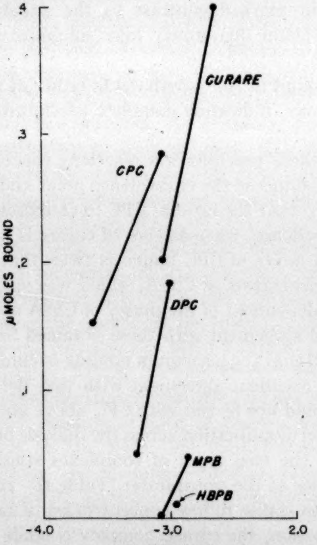

Fig. 4. Binding of various pyridinium derivatives by chondroitin sulfate A as determined by equilibrium dialysis at pH 7.5, ionic strength 0.1, 0°. The binding of curare is shown for comparison with that of HBPB. Binding of the latter is similar to that of MPB, a finding confirmed by the more extensive data of Fig. 5.

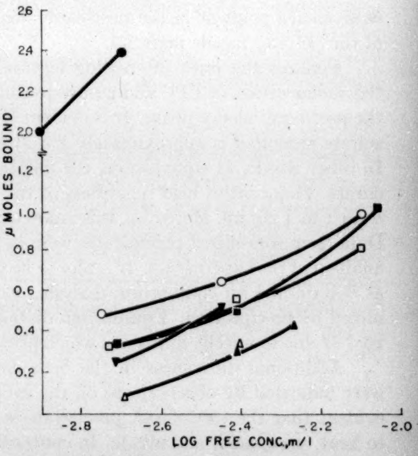

Fig. 5. Binding of various bis-quaternary compounds to ChSA compared with the corresponding mono-quaternary compounds, pH 7.5, ionic strength 0.1, 0°. Data for curare binding under comparable conditions are also shown. ●, curare; ○, HBPB; □, DBPB; △, MPB; ■, curve for both H bis PAM and D bis PAM; ▲, PAM; ▼, hexamethylene PAM.

DISCUSSION

The data based on precipitation and equilibrium dialysis studies indicate that there may be stoichiometric binding between some quaternary ammonium compounds and chondroitin sulfate. Two molecules of CPC, each with a single functional group, react with both functional groups of the repeating unit of ChSA. However, only one molecule of the bifunctional curare is required, suggesting that the inter-quaternary distance in this compound is such as to permit it to attach simultaneously to the two negative groups of the repeating unit. Regardless of the mode of interaction, it is safe to assume that the principal binding force is electrostatic. Thus we find that the precipitated complexes may be dispersed by increasing concentrations of salt. However, this cannot be the only factor involved as indicated by the marked difference in the stability of these complexes to salt, pH, urea, or heat. Thus curare–ChSA precipitates are readily soluble in 0.1 *M* NaCl, whereas CPC–ChSA precipitates are completely soluble only when the concentration of NaCl is increased to 1 *M*. It would appear that at low concentrations of electrolyte, mutual repulsion of the negatively charged groups on ChSA occurs, this apparently providing the proper spatial configuration for the two-point attachment of curare. However, as the ionic strength increases, the configuration of the ChSA may change[17, 18], the two-point attachment of the relatively rigid curare molecule may be broken and there is either a decrease in the amount of curare bound or solubilization. On the other hand, the CPC–ChSA complex would be expected to be less salt sensitive because only single charges are involved. Moreover, there is the additional stabilizing factor due to the VAN DER WAALS' forces exerted through the hydrophobic portion of the long chain compound.

Changes in pH down to pH 3.5 produce effects which are somewhat comparable to changes in electrolyte concentration. As the pH is lowered, ionized carboxyl groups on the ChSA are converted to COOH. Under these conditions, the ChSA would be expected to undergo configurational changes together with a decrease in the number of binding sites. Consequently, the binding of curare is very much reduced whereas the CPC–ChSA complexes are quite stable over a wider range of pH since binding occurs with individual carboxyl and sulfate groups.

The effect of either changes in temperature or the addition of urea point to the contribution of hydrogen bonding toward the stabilization of curare complexes. Thus curare–ChSA precipitates (Table II) are dispersed by warming to 70° or by the addition of urea to a final concentration of 1 *M*. Under similar conditions, CPC precipitates are quite stable. The influence of hydrogen bonding is further substantiated by a comparison of curare with its dimethyl derivative. Both compounds form precipitates with ChSA which are salt-labile, but the affinity of curare for ChSA is much greater than that of the dimethyl derivative; much higher concentrations of the latter are required for precipitation. Similar differences in their binding properties were obtained by equilibrium dialysis experiments. Thus, the phenolic hydroxyl groups of curare, which are present as O-methyl ethers in the methylated derivative, are free to form hydrogen bonds and thereby would be expected to contribute to the binding force. Interestingly, the dimethyl curare–ChSA complex is also dispersed by urea, indicating the possibility that still other hydrogen bonds may be involved.

Many investigators have reported that an increase in the chain length of a quaternary compound enhances its binding to polymeric materials. Our results on the influence of chain length, based on equilibrium dialysis, which appear in Fig. 4, confirm this general conclusion. MPB gives soluble complexes whereas both DPC and CPC produce precipitates; PAM complexes are soluble whereas those formed with its dodecyl derivative are insoluble. With an increase in the chain length of the quaternary compound, the complex becomes more hydrophobic and less water soluble. Preliminary experiments indicate that the six carbon side chain, *e.g.*, on H-PAM, is not sufficiently long to result in the precipitation of ChSA or even to significantly enhance the affinity of the compound over that of PAM itself (Fig. 5). In addition, the longer chains are more likely to aggregate into micelles through VAN DER WAALS' forces, thereby imparting a reduced water solubility. Indeed, it is apparently this tendency of the nonpolar end of the complex to associate through secondary valence forces which accounts for the solubility of the CPC–ChSA complex in solvents such as alcohol or acetone. This is evidently not the case with either the curare or dimethyl curare–ChSA complexes, both of which are insoluble in these solvents.

From our results, it was also evident that a number of di-quaternary compounds, viz., bis-4-PAM, bis-pyridinium, not only failed to give precipitates but did not bind nearly as well as curare. In fact, the binding of these compounds to ChSA was only slightly greater than that of the monoquaternary compound (MPB or PAM). It is to be noted that in solution none of these compounds have the structural rigidity of a molecule such as curare, in which the distance between the positive charges is fixed. This factor is apparently necessary for the formation of a firmly-bound complex. This possibility is supported by observations with stilbamidine, a compound of considerable lower molecular weight and of lesser complexity than curare, but one which also has a rigid structure in solution (Fig. 1). It, like curare, forms insoluble precipitates with ChSA. The nature of the interaction of this compound with ChSA is under investigation.

We may now consider some of the general conclusions which have emerged from these studies regarding the structure of ChSA in solution. The fact that there is a fairly exact equivalence of 1 curare for each 2 negative charges suggests that the negative groups are regularly spaced and that the inter-anionic distance is almost exactly the same as the inter-quaternary distance in curare. The question which may be raised is whether a molecule like ChSA, generally considered to be a random coil in dilute salt solution [17,18], would be expected to have the regular spacing of its negative groups which is required for the type of interaction observed. However, the possibility still exists that the curare molecule itself induces changes in the ChSA configuration such that the negative groups approach the inter-quaternary distance. Two pieces of evidence rule out such a mechanism: (a) Curare failed to alter the viscosity of ChSA and (b) curare failed to precipitate chondroitin sulfate C (CHSC). In the latter polysaccharide, the sulfate group is removed by a few Ångström units from its position in ChSA. Failure of ChS-C to precipitate with curare further substantiates the hypothesis that curare attaches itself to two negative groups and that small differences in the inter-anionic distance results in much reduced interaction. The experiment with ChS-C would appear to provide the best evidence that curare precipitation is not due to inter-molecular binding.

From the evidence presented in this paper, it is apparent that even a very highly anionic polysaccharide such as chondroitin sulfate may play only a minor role *in vivo* insofar as complexing with quaternary ammonium ions is concerned. Of the many such compounds examined, only curare and stilbamidine have an affinity which is sufficiently high to permit interaction with cellular chondroitin sulfate. Other compounds having profound effects when applied to the neuromuscular junction—such as the decamethonium type—interact only weakly. Thus CHAGAS' suggestion[11,12] that acidic polysaccharides may function as "receptors" for various quaternary compounds in general should be modified so as to include only those diquaternary compounds which fit the inter-anionic distance of the polysaccharide repeating unit.

ACKNOWLEDGEMENTS

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REFERENCES

[1] J. E. SCOTT, *Chem. and Ind.*, (1955) 168.
[2] J. E. SCOTT, *Biochim. Biophys. Acta*, 18 (1955) 428.
[3] M. M. FISHMAN AND R. S. MILLER, *J. Colloid Sci.*, in the press.
[4] S. A. BARKER, M. STACEY AND G. ZWEIFEL, *Chem. and Ind.*, (1957) 330.
[5] I. DANISHEFSKY, E. T. OPPENHEIMER, M. WILLHITE, A. P. STOUT AND M. M. FISHMAN, *Cancer Research*, 19 (1959) 1234.
[6] E. D. KORN, *J. Biol. Chem.*, 234 (1959) 1325.
[7] H. G. B. SLACK, *Biochem. J.*, 69 (1958) 125.
[8] S. EHRENPREIS, *Science*, 129 (1959) 1613.
[9] S. EHRENPREIS, *Biochim. Biophys. Acta*, 44 (1960) 561.
[10] S. EHRENPREIS AND M. M. FISHMAN, *Abstracts 135th Meeting, Am. Chem. Soc., April, 1959.*
[11] C. CHAGAS, E. PENNA-FRANCA, K. NISHIE AND E. J. GARCIA, *Arch. Bioch. Biophys.*, 75 (1958) 251.
[12] A. HASSON AND C. CHAGAS, *Biochim. Biophys. Acta*, 36 (1959) 301.
[13] J. EINBINDER AND M. SCHUBERT, *J. Biol. Chem.*, 191 (1951) 591.
[14] E. A. DAVIDSON AND K. MEYER, *J. Am. Chem. Soc.*, 77 (1955) 4796.
[15] I. M. KLOTZ, F. M. WALKER AND R. B. PIVAN, *J. Am. Chem. Soc.*, 68 (1946) 1486.
[16] F. W. PUTNAM, *Advances in Protein Chem.*, Vol. 4, Academic Press, Inc, New York, 1948, p. 80.
[17] M. B. MATHEWS AND A. DORFMAN, *Arch. Biochem. Biophys.*, 42 (1953) 41.
[18] M. B. MATHEWS, *Arch. Biochem. Biophys.*, 43 (1953) 181.

Short Communications

The preparation of crystalline β-methylaspartase

The enzyme β-methylaspartase, which catalyzes the reversible conversion of β-methylaspartate to mesaconate and ammonia, has been isolated and purified some 45-fold from *Clostridium tetanomorphum* by BARKER *et al.*[1]. As the preliminary step in a study of the stereochemistry and mechanism of the reaction catalyzed by this enzyme, β-methylaspartase has been further purified and crystallized in this laboratory. The method of purification and assay of BARKER *et al.*[1] was used, except that DEAE-cellulose chromatography and subsequent crystallization were substituted for the calcium phosphate gel step.

DEAE-cellulose (Brown Company, Berlin, New Hampshire) was stirred into water and allowed to settle. The light particles which floated were decanted and the process repeated until none remained. The DEAE-cellulose was filtered off, stirred thoroughly into satd. KCl, filtered again and stirred into a fresh KCl solution. The DEAE-cellulose was freed of solution on a Buchner funnel. This material was added to a solution of 0.01 *M* potassium phosphate buffer, pH 7.85, and packed under N_2 at 5 lb./in.^{2} to a height of 30 cm in a 2 × 60 cm column which was jacketed for iced water. The column was washed with 200 ml 0.01 *M* potassium phosphate buffer, pH 7.85.

40 ml of a solution of β-methylaspartase in 0.01 *M* potassium phosphate buffer, pH 7.85 (containing 127 mg of protein of specific activity 200) from the ethanol-fractionation step of BARKER *et al.*[1] were placed on the column. The enzyme was eluted with KCl in 0.01 *M* potassium phosphate buffer, pH 7.85. The concentration of KCl was changed in a stepwise manner and 5-ml fractions were collected at the rate of 2 ml/min. The enzyme is displaced by 0.1 *M* KCl under these conditions (see Fig. 1).

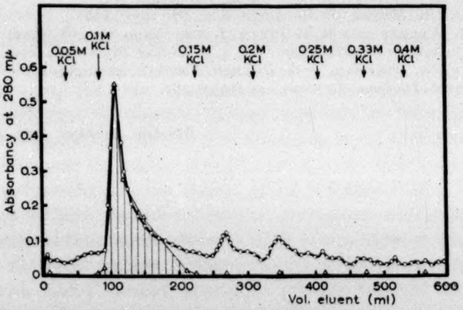

Fig. 1. Chromatography of β-methylaspartase. ○—○, u.v. absorption at 280 mμ. △—△, enzyme activity in arbitrary units. For conditions, see text.

Abbreviations: DEAE-cellulose, diethylaminoethyl-cellulose; EDTA, ethylenediaminetetraacetate.

All active fractions in the peak were combined (140 ml total) and added to 10 ml 1.0 *M* potassium phosphate buffer, pH 7.85. 84 g of ammonium sulfate were then added and the precipitated protein centrifuged off at 18,000 × *g*, at 15°. The precipitate was dissolved in 2 ml 0.01 *M* potassium phosphate buffer, pH 7.85. Satd. ammonium sulfate solution, pH 7.85, was then added slowly until there was incipient cloudiness. The solution was left in the refrigerator overnight after which time small, needle-like crystals could be seen in the microscope. More saturated ammonium sulfate solution was added until no more crystals appeared. The enzyme was once recrystallized. The 2.5-fold purification of β-methylaspartase is shown in Table I.

TABLE I

SUMMARY OF PURIFICATION PROCEDURE

Fraction	*Volume*	*Protein*	*Activity*		*Specific activity*
			Units	*Yield*	
	ml	*mg*	$\times 10^3$	%	*units/mg*
1. Ethanol fraction (see ref. 1)	40	127	25.4	60	200
2. Combined fractions from DEAE-cellulose	140	39	19.1	45	490
3. Product of first crystallization	—	32	15.8	37	494
4. Product of second crystallization	—	26.3	13.1	31	498

The crystals are small and are seen most readily with phase-contrast microscopy (see Fig. 2). They are very stable, retaining full activity at 4° after 1 year. A 1-% solution of the crystals containing 0.10 *M* potassium phosphate buffer, pH 7.05, and 0.2 *M* KCl shows a single and symmetrical peak in the ultracentrifuge corresponding to a sedimentation coefficient of 5 S (see Fig. 3).

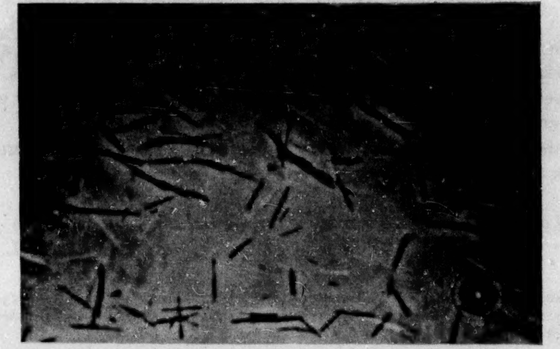

Fig. 2. Crystals of β-methylaspartase (once recrystallized), × 750.

After dialysis against 0.005 *M* EDTA in 0.005 *M* potassium phosphate buffer, pH 7.0, the divalent metal ion requirement of the crystalline enzyme is satisfied by the following under the conditions of the assay:

$$Mg^{++} > Mn^{++} > Co^{++} > Ni^{++}$$

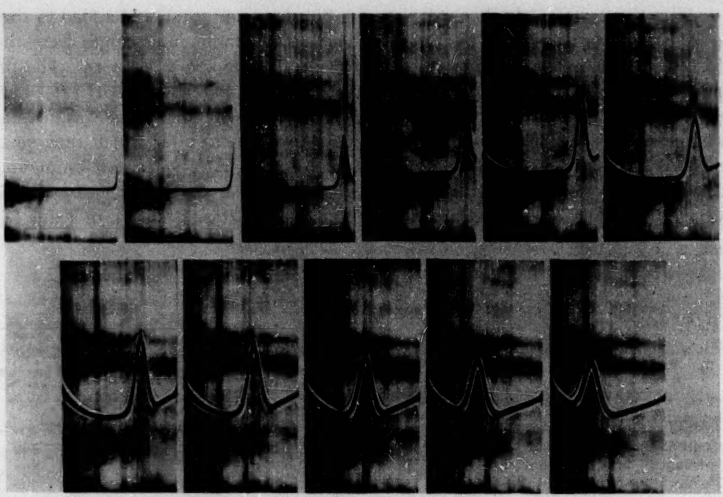

Fig. 3. Sedimentation pattern of β-methylaspartase (1% by weight, once recrystallized) in 0.10 *M* potassium phosphate buffer, pH 7.05, containing 0.2 *M* KCl obtained in the Spinco Model E Ultracentrifuge. Temp. 13.7°, rotor speed 59,780 rev./min. Exposure times 0, 8, 16, 24, 32, 48, 64, 80, 96, 112 and 128 min. Angle of Schlieren diaphragm 80°, 80°, 80°, 70°, 70°, 70°, 60°, 60°, 60°, 60°, and 60°.

Less active preparations are activated only by Mg^{++} (see ref. 1). Also, under conditions where less purified preparations of β-methylaspartase were shown to produce a mixture of β-methylaspartate isomers from mesaconate and ammonia[1], only the *threo* isomer could be detected when the crystalline enzyme was used to catalyze the reaction between mesaconate and ammonia. Paper electrophoresis[1] and nuclear-magnetic-resonance spectroscopy** were used to distinguish the isomers.

However, it has not yet been possible to determine unequivocally whether the crystalline enzyme eliminates ammonia from the *erythro* isomer.

We wish to thank Dr. H. A. BARKER for a gift of *Cl. tetanomorphum* and Dr. H. K. SCHACHMAN for the sedimentation pattern.

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[1] H. A. BARKER, R. D. SMYTH, R. M. WILSON AND H. WEISSBACH, *J. Biol. Chem.*, 234 (1959) 320.
[2] L. BENOITON, S. M. BIRNBAUM, M. WINITZ AND J. P. GREENSTEIN, *Arch. Biochem. Biophys.*, 81 (1959) 434.

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* BARKER *et al.* tentatively assigned the α-L-*threo* configuration to the isomer of β-methylaspartate which is more readily used by β-methylaspartase. BENOITON *et al.* confirmed the α-L-assignment by the action of a stereospecific acylase[2]. We have since proved the *threo* configuration of this isomer by nuclear-magnetic-resonance spectroscopy, the details and implications of which will be published shortly elsewhere.

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Isolation and identification of the orange pigment in the fungus *Cladochytrium replicatum*

Among the uniflagellate Phycomycetes, carotenes occur commonly in several species of *Allomyces*, in *Blastocladiella* sp., and in *Karlingia rosea*. The pigments of these fungi have been discussed by HAXO[1]. Although *Cladochytrium replicatum*, a monocentric chytrid, was first described by KARLING[2] as having "a bright golden brown globule" in its zoospores, no identification of the major orange pigment of this fungus has been published to date. A pure culture of *C. replicatum* was sent to M. S. FULLER in 1956 by Dr. L. G. WILLOUGHBY, Freshwater Biological Station, Ambleside, England. According to WILLOUGHBY, this fungus, although orange, rarely produces sporangia when cultured on agar. Our own observations on both Emerson's YpSs agar (Difco) and CRASEMANN's[3] medium 1B plus Difco yeast extract (1 g/l) confirmed the absence of sporangial production under such conditions. However, the plants were still orange-brown in color with the pigment localized in the rhizomycelial swellings. The present study was undertaken to isolate and identify this pigment.

A suspension of rhizomycelium fragments, prepared by grinding the fungus for 30 sec in a Waring Blendor with distilled water, was used to inoculate Petri dishes containing YpSs agar over which a piece of sterile cellophane dialysis membrane had been placed. After 3 weeks growth at 25°, the fungus was harvested by pulling the dialysis membrane from the agar surfaces. Unfortunately, this latter step was made difficult by the partial decomposition of the cellulose by *C. replicatum*.

Acetone was used to extract the pigment from the rhizomycelium. Following extraction, the pigment was transferred to petroleum ether. This was done by mixing petroleum ether, the acetone extract, and 5 % aq. NaCl in a separatory funnel. The hypophasic acetone–NaCl–water layer was removed and the remaining petroleum ether with the pigment was evaporated by means of a water aspirator to a volume of approx. 2 ml. This pigment in petroleum ether was transferred to a chromatographic column containing magnesium oxide–Johns-Manville Celite 545 (1:2, w/w). This column was developed with suction using petroleum ether containing 1 % acetone by volume.

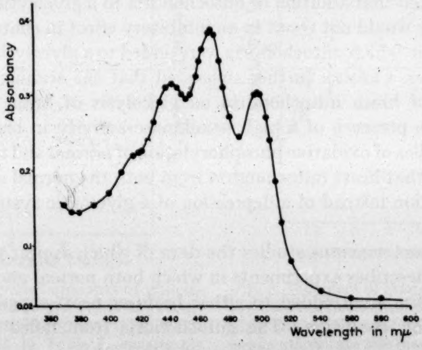

Fig. 1. Absorption spectrum for lycopene extracted from the fungus *Cladochytrium replicatum* and dissolved in petroleum ether.

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Following development, only one pigmented red-orange band was present. This band was removed from the column and transferred as above to pure petroleum ether. Identification of the pigment was made by measuring the light absorption at 2-mμ intervals in wavelength from 375–700 mμ with a Beckman DU spectrophotometer. Fig. 1 shows the absorption curve obtained for the pigment isolated from *C. replicatum*. The absorption maxima are at 443, 468, and 498 mμ, and the curve is a characteristic one for the carotene, lycopene. TURIAN AND HAXO published a similar curve with absorption peaks at 445, 470, and 500 mμ for lycopene which they extracted from the gametophytic plants of *Allomyces javanicus*. The authors are indebted to Dr. L. J. WILLOUGHBY for making this isolate of *C. replicatum* available for their study.

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[1] F. T. HAXO, *Fortschr. Chem. Organ. Naturstoffe*, 12 (1955) 169.
[2] J. S. KARLING, *Am. J. Botany*, 18 (1931) 526.
[3] J. M. CRASEMANN, *Am. J. Botany*, 41 (1954) 302.
[4] G. TURIAN AND F. T. HAXO, *Botan. Gaz.*, 115 (1954) 254.

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The effect of heart mitochondria on glycolytic systems from brain and heart

In recent years a number of investigators[1,2] have utilized the Pasteur effect in attempts to elucidate regulatory mechanisms involved in carbohydrate metabolism. Among these have been the studies of AISENBERG, REINAFARJE AND POTTER[3], AISENBERG[4] and more recently, CREMER[5]. AISENBERG *et al.* found that liver mitochondria inhibited glycolysis of a brain supernatant system, while CREMER described an inhibitory or stimulatory effect of rat-liver and -brain mitochondria, respectively, on the same system.

CREMER indicated that addition of mitochondria to a glycolytic system prepared from the same tissue would not result in an inhibitory effect in contrast to the studies by AISENBERG *et al.* in which mitochondria were added to a glycolytic system obtained from different tissues. CREMER further suggested that the stimulatory, rather than inhibitory, action of brain mitochondria on glycolysis of brain supernatant was probably due to the presence of a high hexokinase activity in brain mitochondria.

During our studies of oxidative phosphorylation of normal and failed guinea pigs[6], it became apparent that heart mitochondria from both the normal and failed animals produced a stimulation instead of a depression of a glycolytic system obtained from brain tissue.

The present report concerns studies the data of which appear to be pertinent to the above work. It describes experiments in which both normal and "failed" guinea-pig-heart mitochondria were added to either brain-or heart-supernatant glycolytic systems under aerobic conditions. The mitochondria from failed guinea pigs were

---

Abbreviations: ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetate.

obtained from animals in which experimental congestive heart failure was produced. These mitochondria were severely uncoupled with respect to oxidative phosphorylation. The normal heart and liver mitochondria were obtained from healthy guinea pigs and showed normal P:O ratios. In all cases a marked stimulation of glycolytic rate by the heart mitochondria was observed.

TABLE I

THE EFFECT OF MITOCHONDRIA ISOLATED FROM NORMAL GUINEA-PIG LIVER AND HEART AND FROM FAILED HEART ON AEROBIC GLYCOLYSIS OF BRAIN-SUPERNATANT SYSTEM

Glycolytic activity of the guinea-pig-brain supernatant (S_3) was measured by the disappearance of glucose[7] as well as the production of lactic acid[8]. The glycolytic medium contained 0.006 *M* glucose, 0.6 m*M* ATP, 0.009 m*M* DPN, 0.025 *M* $KHCO_3$; and 0.004 *M* $MgCl_2$ in a total volume of 3.0 ml. The pH was adjusted to 7.4. Incubation was carried out in a Dubnoff Metabolic Shaker at 38° for 30 min in an atmosphere of O_2–CO_2 (95:5). Each flask contained 1 ml of mitochondrial suspension (1:1 dilution with 0.16 *M* KCl or 0.25 *M* sucrose with 0.001 *M* EDTA and containing 3–5 mg protein/ml) and 0.6 ml supernatant (S_3) representing approx. 240 mg of original tissue. All values (average of 4 expts.) are changes during 30-min incubation.

Mitochondria	*+ Δ Lactate (μmoles)*		*— Δ glucose (μmoles)*	
	S_3 alone	*Mitochondria + S_3*	*S_3 alone*	*Mitochondria + S_3*
Liver	4.53	0.04	—	—
	5.96	2.78	5.50	4.00
Normal heart	6.60	15.75	6.10	12.80
	3.42	5.08	4.00	5.70
	2.80	6.30	3.40	7.20
	2.10	3.00	2.70	3.70
Failed heart	1.41	4.70	3.00	5.90
	3.75	8.80	3.70	6.20
	3.60	6.15	3.40	3.80
	3.29	7.26	3.40	5.60

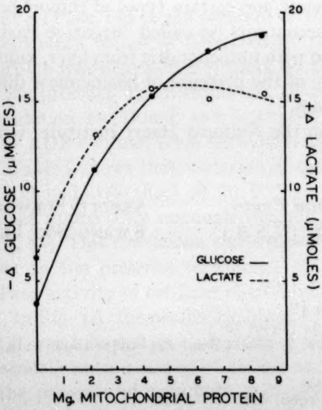

Fig. 1. The effect of graded amounts of guinea-pig-heart mitochondria on glycolysis of guinea-pig-heart supernatant. The glycolytic medium was the same as that in Table I, except for the addition of 40 μmoles potassium phosphate buffer, pH 7.4, to each flask.

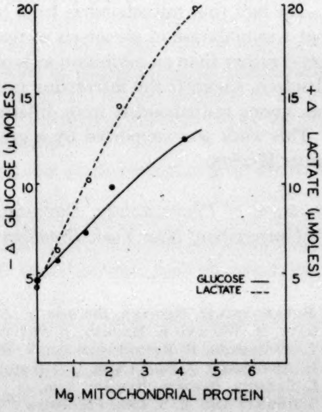

Fig. 2. The effect of graded amounts of guinea-pig-heart mitochondria on glycolysis of guinea-pig-brain supernatant. Glycolytic rate is expressed in μmoles glucose disappeared and in μmoles lactic acid produced. The glycolytic medium and incubation conditions were the same as in Table I.

The results presented in Table I show that guinea-pig-liver mitochondria, like the rat-liver preparations of AISENBERG *et al.*, effected a marked inhibition of glycolysis of brain supernatant. On the other hand, both normal and uncoupled heart mitochondria, doubled the rate of glycolysis. The relative amounts of mitochondria, glycolytic medium and the supernatant were similar to those employed by AISENBERG and by CREMER. The latter author, however, included inorganic phosphate while the former and the present authors did not. Inorganic phosphate increases the absolute rate of glycolysis but does not alter the stimulating effect of the heart mitochondria, as is indicated in Fig. 1.

The results plotted in Figs. 1 and 2 show that the stimulating effect of heart mitochondria on brain or heart supernatant is graded, *i.e.* with increasing amounts of mitochondrial protein, there is an increasing stimulation on glycolysis.

The addition of heart mitochondria to a glycolytic system prepared from the same tissues does not affect the results, as indicated in Figs. 1 and 2, where the stimulating effect of heart mitochondria is seen with either brain or heart supernatant (the glycolytic supernatant from heart was prepared in the same way as from brain[3]). Mitochondria isolated from severely failed guinea-pig hearts whose P:O ratio was reduced to less than 50 % of the control values still bring about the same type of stimulating action on glycolysis (Table I). This is in agreement with the data of AISENBERG *et al.* who found that uncoupling of liver mitochondria did not alter their results, and is in keeping with their suggestion that the Pasteur effect observed in this type of system is not explainable on the basis of a mitochondrial competition with glycolytic system for available inorganic phosphate or phosphate acceptor.

The stimulating effect of heart mitochondria on glycolysis is not explained by the presence of hexokinase as in brain, since heart mitochondria appear to contain little or no hexokinase activity (unpublished data).

The fact that mitochondria from brain, heart and certain types of tumor[3] bring about a stimulation of glycolysis of tissue supernatants (so called "negative Pasteur effect") rather than an inhibition as is observed with mitochondria from liver, kidney, and spleen, suggests the interesting possibility of the presence of biochemical differences among mitochondria from different tissues.

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[1] R. BALAZS AND D. RICHTER, *Biochem. J.*, 68 (1957) 5P.
[2] S. GATT, R. WU AND E. RACKER, *J. Biol. Chem.*, 234 (1959) 1015.
[3] A. C. AISENBERG, B. REINAFARJE AND V. R. POTTER, *J. Biol. Chem.*, 224 (1957) 1099, 1115.
[4] A. C. AISENBERG, *J. Biol. Chem.*, 234 (1959) 441.
[5] J. E. CREMER, *Biochim. Biophys. Acta*, 41 (1960) 155.
[6] A. SCHWARTZ AND K. S. LEE, *Federation Proc.*, 19 (1960) 116.
[7] S. B. BARKER AND W. H. SUMMERSON, *J. Biol. Chem.*, 138 (1941) 535.
[8] A. SAIFER AND S. GERSTENFELD, *J. Lab. Clin. Med.*, 51 (1958) 448.

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Measurement of the P:O ratio associated with the oxidation of ascorbate by rat-liver mitochondria

It is the purpose of the present report to draw attention to a source of error which can arise under certain circumstances in the measurement of the P:O ratio associated with the oxidation of ascorbate by rat-liver mitochondria. This is caused by the presence of appreciable amounts of endogenous substrate in these mitochondrial preparations[1].

The experiments described were carried out with rat-liver mitochondria isolated by the method of HOGEBOOM[2], exactly as described by MYERS AND SLATER[3]. The suspension in 0.25 *M* sucrose was diluted 3.3-fold with ice-cold distilled water, to give a suspension medium of 0.075 *M* sucrose (see ref. 4) and allowed to stand 10 min at 0° before adding to the reaction medium. The final composition of the reaction medium (volume 1 ml) was 25 m*M* sucrose, 10 m*M* potassium phosphate, 30 m*M* glucose, 0.5 m*M* ADP, 5 m*M* $MgCl_2$, 2 m*M* EDTA, 20–50 m*M* ascorbate (freshly prepared), 100–150 units[5] crystalline yeast hexokinase[6], and varying amounts of cytochrome *c*. The final pH was 6.5. When ascorbate was omitted, 40 m*M* sucrose was added to maintain the tonicity of the solution. When antimycin was added, the reaction mixture contained 0.5 % ethanol derived from the antimycin solution. The cytochrome *c*, prepared by the method of MARGOLIASH[7], had $A_{550\,m\mu}$ (reduced)/$A_{280\,m\mu}$ (oxidized) of 1.22 or higher. Oxidative phosphorylation was measured by the method of SLATER[8]. The O_2 uptake was measured with differential manometers. The constants, calculated according to VAN DORP AND SLATER[9], were between 0.25 and 0.70 μl O_2/mm manometer fluid. The O_2 uptake was corrected for that found in a control experiment, in which mitochondria were omitted.

The hypotonic pretreatment of the mitochondria caused some development of latent ATPase, but in the presence of Mg^{++} dinitrophenol still stimulated the ATPase 12-fold when tested as described by GREENGARD *et al.*[10]. The rate of oxidation of ascorbate was increased 2–3 times by the addition of phosphate or of dinitrophenol. (In the presence of dinitrophenol, phosphate caused no further increase of the rate of oxidation–see BORST AND SLATER[11]). The P:O ratio was not affected by variation of the ADP concentration between 0.24 and 3.2 m*M*.

Table I shows that variation of the cytochrome *c* concentration between 10 and 250 μM had little effect on the P:O ratio. Thus, high concentrations of cytochrome *c* did not appreciably uncouple oxidative phosphorylation under our conditions (see ref. 4, 12). This conclusion was supported by the finding that 100 μM cytochrome *c*, either in the presence or absence of 10^{-4} *M* dinitrophenol, had no effect on the ATPase activity of rat-liver mitochondria (see ref. 3).

In Fig. 1A, the results are given of an experiment, in which the amount of esterified P was followed as a function of time, both in the absence (curves 1 and 3) and presence (curves 2 and 4) of antimycin. Curves 1 and 2 describe the results obtained in the presence of ascorbate and cytochrome *c*, curves 3 and 4 the corresponding results in the absence of substrate. Curve 3 shows that there was considerable esterification of phosphate when the mitochondria were incubated in the reaction medium, in the absence of added substrate, much of the esterification occurring in the first

---

Abbreviations: ADP, ATP, adenosine diphosphate and triphosphate; EDTA, ethylenediamine tetraacetic acid.

TABLE I

EFFECT OF CYTOCHROME C CONCENTRATION ON P:O RATIO OF RAT-LIVER MITOCHONDRIA

Conditions as described in text; 0.45–0.65 μg antimycin/mg protein.

Cytochrome c (*μM*)	*P:O*		
	Expt. 1		*Expt. 2*
Ascorbate (mM)	20	50	20
9	0.51	0.62	0.46
12	0.54	0.59	0.45
22		0.64	0.46
43	0.54	0.65	0.45
62	0.45	0.59	0.46
124	0.46	0.55	
248	0.45	0.54	

5 min. It was abolished by addition of 0.45 μg antimycin/mg protein (curve 4), an amount of antimycin which was sufficient to inhibit the oxidation of succinate by 98 %. This esterification of phosphate in the absence of substrate is very likely due to endogenous substrates present in the mitochondria[1].

Since oxidative phosphorylation associated with the oxidation of endogenous substrates has a higher P:O ratio than that associated with the oxidation of ascorbate[13], the latter P:O will be over-estimated unless interference due to oxidation of endogenous substrate is eliminated or allowed for. With the method used in this study to measure the O_2 uptake, the error is even greater than it appears, because most of the extra O_2 uptake occurs in the first 5 min, and is not detected or allowed for in our procedure in which the uptake in the first 5 min is calculated by extrapolation, assuming a constant oxidation rate. The rate of phosphorylation in the first 2 min is about double that between 5 and 25 min. This could give the impression that there is a very labile phosphorylation step associated with the oxidation of ascorbate

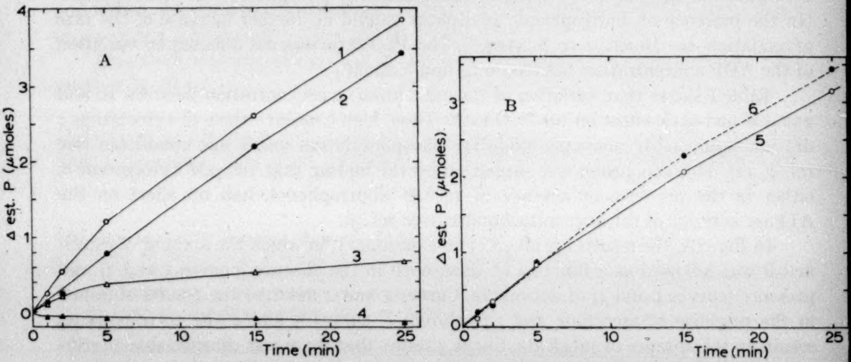

Fig. 1. Phosphorylation coupled to oxidation of endogenous substrate and to oxidation of added ascorbate. Protein, 2.20 mg/ml. A. Curve 1, ascorbate, 0.02 *M*; cytochrome *c*, $2.2 \cdot 10^{-5}$ *M*; curve 2, same as curve 1 with addition of antimycin (0.45 μg/mg protein); curve 3, no cytochrome *c* or ascorbate; curve 4, same as 3 with addition of antimycin (0.45 μg/mg protein). B. Curve 5, curve 1 of A *minus* curve 3; curve 6, curve 2 of A *minus* curve 4.

and, moreover, that this labile step is sensitive to antimycin (*cf.* curves 1 and 2, Fig. 1A).

When, however, the amount of esterified P found in the absence of ascorbate and cytochrome *c* is subtracted from that found in the presence of substrate, practically identical values were obtained in the presence and absence of antimycin (Fig. 1B), and there was little fall in oxidative phosphorylation during the course of the experiment.

Unless oxidation of endogenous substrate is inhibited by the addition of antimycin, or a suitable correction is made, the measured P:O ratio will be increased whenever conditions are altered in such a way as to increase the proportion of the total O_2 uptake which is concerned with the oxidation of endogenous substrate, *e.g.* by running the experiment for a short time, or by choosing conditions unfavourable to the oxidation of ascorbate, such as the use of mitochondria which have not been subjected to hypotonic pretreatment. The finding of increased P:O ratios under these conditions will lead to the conclusion, which might be incorrect, that these conditions favour the phosphorylating step in the cytochrome oxidase region of the respiratory chain.

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[1] K. MINNAERT, *Biochim. Biophys. Acta*, 44 (1960) 595.
[2] G. H. HOGEBOOM, *Methods in Enzymology*, Vol. I, Academic Press, New York 1955, p. 16.
[3] D. K. MYERS AND E. C. SLATER, *Biochem. J.*, 67 (1957) 558, 572.
[4] A. L. LEHNINGER, M. HASSAN AND H. C. SUDDUTH, *J. Biol. Chem.*, 210 (1954) 911.
[5] L. BERGER, M. W. SLEIN, S. P. COLOWICK AND C. F. CORI, *J. Gen. Physiol.*, 29 (1946) 379.
[6] R. A. DARROW AND S. P. COLOWICK, personal communication.
[7] E. MARGOLIASH, *Biochem. J.*, 56 (1954) 529, 535.
[8] E. C. SLATER, *Biochem. J.*, 53 (1953) 157. 521.
[9] A. VAN DOPP AND E. C. SLATER, *Biochim. Biophys. Acta*, 33 (1959) 559.
[10] P. GREENGARD, K. MINNAERT, E. C. SLATER AND I. BETEL, *Biochem. J.*, 73 (1959) 637.
[11] P. BORST AND E. C. SLATER, *Nature*, 184 (1959) 1396.
[12] C. COOPER AND A. L. LEHNINGER, *J. Biol. Chem.*, 219 (1956) 519.
[13] B. CHANCE AND G. R. WILLIAMS, *J. Biol. Chem.*, 217 (1955) 383.

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Endogenous respiration of rat-liver mitochondria*

The presence of appreciable amounts of endogenous substrate in rat-liver mitochondria was first reported by CHANCE AND WILLIAMS[3]. During the course of measurements of the phosphorylation coupled to oxidation of ascorbate in the presence of cytochrome *c* and rat-liver mitochondria, we found that the simultaneous oxidation of

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Abbreviations: ADP, adenosine diphosphate; EDTA, ethylenediaminetetraacetic acid.

* This paper and the previous[2] is part of the Ph.D. thesis of the author, published in Dutch[1]. It was defended in public on June 22nd 1960, before the Senate of the University of Amsterdam.

endogenous substrate was a disturbing factor[1,2]. This paper reports a further study of the rate of oxidation of endogenous substrate and of the accompanying phosphorylation.

The oxidation of endogenous substrate was followed by measuring either the oxygen uptake or the esterification of inorganic phosphate. The experimental methods were the same as those described in the accompanying paper[2]. The "standard reaction mixture" consisted of phosphate, 10 m*M*; glucose, 30 m*M*; ADP, 0.6 m*M*; $MgCl_2$, 5 m*M*; EDTA, 2 m*M*; sucrose, 65 m*M*; and hexokinase, 100–150 units. The reaction mixture was brought to pH 6.5. The mitochondria were subjected to a hypotonic treatment (7.5 m*M* sucrose) for 10 min at 0°.

The esterification of inorganic phosphate during aerobic incubation of the mitochondria in the reaction mixture is a measure of the oxidation of substrates inside the mitochondria or adsorbed on their surface. The hypotonic pretreatment of the mitochondria or an extra washing with 0.25 *M* sucrose, had little effect on the amount of endogenous substrate measured in this way (see Fig. 1).

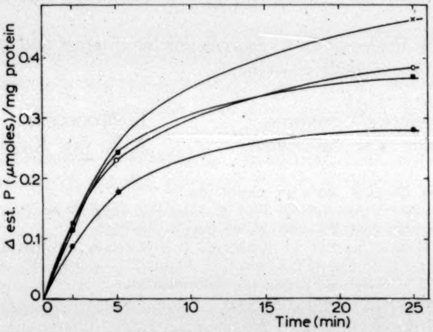

Fig. 1. Effect of hypotonic pretreatment and of washing on amount of endogenous substrate in rat-liver mitochondria. ×, normal mitochondria; ○, normal mitochondria given extra wash; ■, hypotonically pretreated mitochondria (normal preparation used in this study); ●, mitochondria given extra wash and then hypotonically pretreated.

The amount of phosphate esterified in the first 5 min was 0.20 μmole/mg protein (average of 10 experiments; range, 0.14–0.25). After 5 min the rate falls off; over the first 25 min, 0.36 μmole P/mg protein were esterified (average of 7 experiments; range, 0.28–0.46). If we assume a P:O ratio of 2, this corresponds to a Q_{O_2} of 13.5 during the first 5 min and of 2.6 during the following 20 min. It is not possible to indicate the total amount of endogenous substrate since the oxygen uptake does not come to an end even after several hours.

The maximal initial rate of phosphorylation is obtained with the standard reaction mixture. When ADP was omitted, the initial rate was much slower and a burst of activity equal to the initial rate was found when ADP was added after 5.5 min (Fig. 2). This shows that the concentration of ADP limits the rate of phosphorylation in the presence of phosphate, hexokinase and glucose.

The oxygen uptake by endogenous substrates is inhibited by malonate (see ref. 3, 4) and arsenite. Monoiodoacetate is also inhibitory. The degree of inhibition by this compound (25 % at 0.2 m*M* and 70 % at 1 m*M*) points to an inhibition in the Krebs cycle, rather than in the glycolytic reactions[5]. The preparation of serum albumin used (Armour Laboratories, fraction V) increased the oxygen consumption after 5 or 25 min by a factor of 4, when 10 mg/ml were added to the reaction mixture. However, the supernatant of boiled serum albumin had the same effect while dialysed serum albumin was inactive.

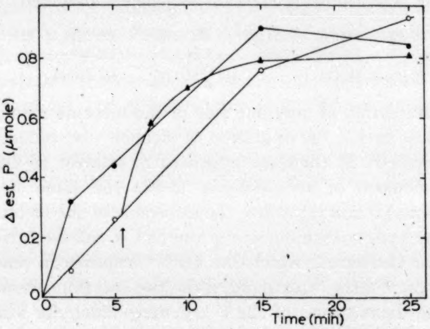

Fig. 2. Effect of ADP on phosphorylation coupled to oxidation of endogenous substrate. ●, standard reaction mixture; ○, ADP omitted; ▲, ADP added after 5.5 min.

The amount of oxygen taken up in 4 h was about 0.75 μatom/mg protein or 75 μatoms/ml wet wt. mitochondrial pellet. If we assume that the endogenous substrate is fatty acid[4] which is oxidized to CO_2 and H_2O, this amount of oxygen is equivalent to a concentration of 1.5 m*M* stearic acid in the pellet. Since it is unlikely that such high concentrations of a substrate are present in mitochondria, it seems probable that the endogenous substrate is continuously formed, *e.g.* by hydrolysis of lipid.

We wish to thank Prof. Dr. E. C. SLATER for his valuable criticism, Miss W. NUWENHOF for excellent technical assistance and Mrs. A. R. VAN KAMMEN-WERTHEIM for cooperation in some of the experiments.

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[1] K. MINNAERT, *Over cytochroom* c *oxydase*, Academic thesis, Amsterdam, 1960. V.R.B. Kleine der A 3-4, Groningen, 1960.
[2] K. MINNAERT AND A. R. VAN KAMMEN-WERTHEIM, *Biochim. Biophys. Acta*, 44 (1960) 593.
[3] B. CHANCE AND G. R. WILLIAMS, *J. Biol. Chem.*, 217 (1955) 383.
[4] M. KLINGENBERG, W. SLENCZKA AND E. RITT, *Biochem. Z.*, 332 (1959) 47.
[5] W. C. YANG, *Science*, 125 (1957) 1087.

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The stereospecificity of the oxidative reaction in the alcohol-dehydrogenase-catalyzed dismutation of aldehydes

Highly purified crystalline horse-liver alcohol dehydrogenase catalyzes the dismutation of formaldehyde and other aldehydes to the corresponding acid and alcohol[1,2]. Evidence has been presented that alcohol dehydrogenase is the only enzyme required for this dismutation[2]. The following reaction sequence is involved:

$$\begin{array}{llr} CH_2O + DPN^+ + H_2O \rightarrow HCOOH + DPNH + H^+ & & (1) \\ CH_2O + DPNH + H^+ \rightarrow CH_3OH + DPN^+ & & (2) \\ \hline 2CH_2O + H_2O \rightarrow CH_3OH + HCOOH & & (3) \end{array}$$

The exclusive utilization of only one side of the nicotinamide ring of DPN+ by alcohol dehydrogenase during the oxidation of alcohols and reduction of aldehydes has been well established[3]. It therefore appeared of interest to determine whether reaction (1), the oxidation of an aldehyde, shows the same stereospecificity as reaction (2). If reactions (1) and (2) utilize the same side of the nicotinamide ring, the dismutation of an aldehyde containing deuterium on C-1 will not introduce deuterium into DPN+, since the deuterium which the DPN+ acquires in reaction (1) will be removed in reaction (2). On the other hand, if the two reactions show opposite stereospecificities, then the dismutation of the C-1 deutero-aldehyde will result in introduction of deuterium into DPN+.

We carried out the dismutation of CH_3CDO in the presence of DPN+ and alcohol dehydrogenase. CH_3CDO was synthesized according to published procedures[4]. Alcohol dehydrogenase was purchased from Worthington Biochemical Company. The dismutation reaction was carried out as described previously[2]. The following concentrations of reactants were used: DPN+, $7.9 \cdot 10^{-4} M$; enzyme, $6 \cdot 10^{-7} M$; acetaldehyde, $7.2 \cdot 10^{-3} M$. The total volume of the reaction mixture was 20 ml. In order to maintain a nearly constant reaction rate an additional 80 μmoles of acetaldehyde were added after 25 min. Under these conditions, the rate of acid formation was maintained between 1.1 and 1.5 μmoles/min. The reaction was allowed to proceed for 60 min; 78.4 μmoles of acid were produced during this time. The nicotinamide portion of the DPN+ was then isolated[5] and its deuterium content determined. No deuterium above the naturally occurring amount could be detected. These results, therefore, indicated that both the alcohol-dehydrogenase-catalyzed oxidation (1) and reduction (2) utilize the same side of the nicotinamide ring of DPN+.

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[1] L. P. Kendal and A. N. Ramanathan, *Biochem. J.*, 52 (1952) 430.
[2] R. H. Abeles and H. A. Lee, *J. Biol. Chem.*, 235 (1960) 1499.
[3] B. Vennesland and F. H. Westheimer, *A Symposium on The Mechanism of Enzyme Action*, Johns Hopkins Press, Baltimore, Maryland, 1954.
[4] F. A. Loewus, F. H. Westheimer and B. Vennesland, *J. Am. Chem. Soc.*, 75 (1953) 5018.
[5] B. Vennesland, A. Marcus and J. R. Stern, *J. Biol. Chem.*, 233 (1958) 722.

Abbreviation: DPN+, DPNH, oxidized and reduced diphosphopyridine nucleotide.

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The biochemistry of methanol poisoning

I. Phosphorylation coupled to the mitochondrial oxidation of formaldehyde

It is now generally accepted that the retinal degeneration associated with methanol ingestion is caused by its metabolic derivative, formaldehyde, and that only primates exhibit this ocular toxicity[1]. During the course of comparative studies on the metabolism of formaldehyde by monkey and beef liver and retinal preparations, it was observed that liver mitochondria are able to catalyze the oxidation of formaldehyde and that this oxidation is accompanied by the esterification of inorganic phosphate. Previously, BERNHEIM[2] had described the oxidation of formaldehyde by rat-liver homogenates and WALKENSTEIN AND WEINHOUSE[3] had demonstrated that various aldehydes, including formaldehyde, are oxidized by rat-liver mitochondria, but none of these investigators studied concomitant phosphate esterification. This communication describes an examination of phosphorylation efficiencies during the oxidation of formaldehyde by mitochondrial fractions of liver and retina.

Thrice-washed mitochondria were isolated from fresh liver tissue of the monkey (*Macaca mulatta*) and the cow using ice-cold 0.25 *M* sucrose, according to the technique of SCHNEIDER AND HOGEBOOM[4]. Beef and monkey retinal mitochondria were isolated in 0.25 *M* sucrose as described by KINI AND COOPER[5].

In accord with the results of earlier workers, we observed that a concentration of formaldehyde higher than 1 m*M* inhibited the respiration of liver mitochondria. However, at concentrations of 1 m*M* or less, incubation of formaldehyde with liver mitochondria resulted in a rapid oxidation of the compound, with a concomitant esterification of inorganic phosphate, giving a P/O ratio of approx. 2 (Table I). From the figures indicating the amount of formaldehyde converted to CO_2, it is apparent that most of the added formaldehyde was oxidized only to formate.

This esterification of inorganic phosphate coupled to formaldehyde oxidation was sensitive to the presence of inhibitors or uncoupling agents such as antimycin A and dinitrophenol. The addition of 1 m*M* amobarbital, an inhibitor at the DPNH-cytochrome *c* reductase level[6], reduced both oxygen uptake and the P/O ratio by about 50 %.

Both DPN and GSH are coenzymes for the formaldehyde dehydrogenase described by STRITTMATTER AND BALL[7]. However, addition of these compounds, either singly or in combination, to liver mitochondria had no significant effect on either the oxygen uptake or the esterification of phosphate.

The electron-transport chain involved in formaldehyde oxidation by liver mitochondria is not clear. From the figures on P/O ratios obtained experimentally, which point to a value of 3, and from the sensitivity of the system to amobarbital, it would appear that the enzyme system involved may be the particulate DPN-linked formaldehyde dehydrogenase. Although the apparent lack of stimulation upon addition of DPN and GSH might appear to oppose this hypothesis, it is known that liver mitochondria can carry DPN-linked oxidations without the necessity of adding coenzyme[8].

Abbreviations: DPN, diphosphopyridine nucleotide; GSH, reduced glutathione; ATP, adenosine triphosphate; DPNH, reduced diphosphopyridine nucleotide.

Mitochondria isolated from the liver of both monkey and cow exhibited the same ability to oxidize formaldehyde through a phosphorylating pathway (Table I). However, beef retinal mitochondria, in contrast to liver mitochondria, were unable

TABLE I

OXIDATION OF FORMALDEHYDE BY LIVER MITOCHONDRIA

Each Warburg flask contained the following basic medium in a final volume of 3.0 ml: sodium phosphate buffer (pH, 7.4), 0.01 *M*; KF, 0.01 *M*; ATP, 0.001 *M*; H^{14}CHO, 0.001 *M* (91,000 counts/min/μmole); MgCl$_2$, 0.067 *M*; KCl, 0.033 *M*; cytochrome *c*, $1.3 \cdot 10^{-5}$ *M*; D-glucose, 0.02 *M*; yeast hexokinase, 7.1 units, assayed by the procedure of CRANE AND SOLS[9]; suspension of liver mitochondria containing 6.4 mg protein, estimated according to LOWRY *et al.*[10]. Incubation temperature, 25°. The reaction was stopped after 45 min by addition of 1.0 ml 10 % trichloroacetic acid. Phosphate uptake was measured by the method of FISKE AND SUBBAROW[11]. Formaldehyde disappearance was estimated by the method of TANENBAUM AND BRICKER[12]. Labeled CO$_2$ in the alkali of the center well was determined by a modification of the method of VILLEE AND HASTINGS[13]. Endogenous oxygen uptake amounted to 1.1 μatoms and was not subtracted from the figures quoted below.

Special additions	*Oxygen uptake (μatoms)*	*Phosphate esterified (μmoles)*	*P/O ratio*	*HCHO utilized (μmoles)*	*HCHO oxidized to CO$_2$ (μmoles)*
Monkey liver					
None	4.1	7.9	1.95	2.42	0.0083
Antimycin A (1 μg)	0	0	0	0	0.0076
Dinitrophenol (0.1 m*M*)	2.60	— 1.4	0	2.33	0.0102
Amobarbital (1 m*M*)	1.91	1.9	1.0	2.25	0.0072
GSH (0.67 m*M*)	2.75	5.9	2.14	2.14	0.0100
GSH (0.67 m*M*) + DPN (0.67 m*M*)	3.6	7.9	2.17	2.17	0.0092
Beef liver					
None	3.75	5.44	1.44	2.35	0.0142

TABLE II

OXIDATION OF FORMALDEHYDE BY RETINAL MITOCHONDRIA

The experimental conditions were identical to those given in Table I, except that variable amounts of H^{14}CHO (91,000 counts/min/μmole) were added to the medium containing either beef retinal mitochondria (3.4 mg protein) or monkey retinal mitochondria (2.1 mg protein).

Additions	*Oxygen uptake (μatoms)*	*Phosphate esterified (μmoles)*	*P/O*	*HCHO utilized (μmoles)*
Beef retina	0	0	0	0.00
HCHO (0.1 m*M*)				
HCHO (0.2 m*M*)	0	0	0	— 0.01
HCHO (0.5 m*M*)	0	0	0	— 0.05
HCHO (1 m*M*)	0.25	0	0	— 0.21
HCHO (1 m*M*) + DPN (0.67 m*M*)	0.38	0	0	0.063
HCHO (1 m*M*) + GSH (0.67 m*M*)	0.38	0	0	0.25
HCHO (1 m*M*) + DPNH (0.67 m*M*) + GSH (0.67 m*M*)	0.88	0	0	0.94
Monkey retina				
HCHO (1 m*M*) + DPN (0.67 m*M*) + GSH (0.67 m*M*)	0	0	0	1.04
Pyruvate (10 m*M*) + DPN (0.67 m*M*) + fumarate (0.67 m*M*)	5.46	13.2	2.42	—

to oxidize exogenous formaldehyde, added at concentrations ranging from 0.1 to 1 m*M* (Table II). The same preparations, with pyruvate as substrate and fumarate as "sparker", gave a P/O ratio approaching 3, while with succinate as substrate the ratio was 2. Although it could be shown by sensitive isotopic methods that very small amounts of added formaldehyde are oxidized to CO_2, conventional manometric and chemical methods failed to indicate any oxidation of formaldehyde; indeed, after incubation, all the formaldehyde could be recovered quantitatively within the limits of experimental error.

In order to obtain maximal oxidation rates with a substrate such as pyruvate, DPN must be added to the mitochondria of retina, as with those of brain and certain tumors[5,9]. The addition of DPN or GSH had a small and variable effect on formaldehyde utilization, but a combination of these two compounds definitely stimulated the metabolism of formaldehyde. However, the formaldehyde was oxidized by a non-phosphorylating pathway, since no esterification of inorganic phosphate could be demonstrated (Table II).

To determine whether the retina of a primate exhibited any difference from that of the cow in its capacity to utilize formaldehyde, similar experiments were carried out with monkey retina. In the isolation of monkey retinal mitochondria, no attempt was made to exclude choroidal tissues. As seen from Table II, these mitochondria gave essentially the same results as was obtained with those of the cow.

In a forthcoming paper[5] it will be shown that formaldehyde is an extremely potent uncoupler of oxidative phosphorylation, in both beef and monkey retinal mitochondria. Thus, it may be inferred that the ocular toxicity resulting from methanol ingestion, which is noted only in primates, is not based on a difference between primates and non-primates with respect to the retinal metabolism of formaldehyde. Rather, the ocular toxicity may reflect a difference in the rate of production of formaldehyde from methanol and perhaps its subsequent elimination.

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[1] A. P. GILGER, A. M. POTTS AND I. S. FARKAS, *Am. J. Ophthalmol.*, 42 (1956) 244.
[2] F. BERNHEIM, *J. Biol. Chem.*, 186 (1950) 225.
[3] S. S. WALKENSTEIN AND S. WEINHOUSE, *J. Biol. Chem.*, 200 (1953) 515.
[4] W. C. SCHNEIDER AND G. H. HOGEBOOM, *J. Biol. Chem.*, 183 (1950) 123.
[5] M. M. KINI AND J. R. COOPER, in preparation.
[6] B. CHANCE, in O. H. GAEBLER, *Enzymes*, Academic Press, New York, 1956, p. 447.
[7] P. STRITTMATTER AND E. G. BALL, *J. Biol. Chem.*, 213 (1953) 445.
[8] R. K. CRANE AND A. SOLS, in S. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, N.Y., 1955, p. 277.
[9] S. WEINHOUSE, *Advances in Cancer Research*, 3 (1955) 269.
[10] O. H. LOWRY, N. J. ROSEBOROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
[11] C. H. FISKE AND Y. SUBBAROW, *J. Biol Chem.*, 81 (1929) 629.
[12] C. G. TANENBAUM AND C. E. BRICKER, *Anal. Chem.*, 23 (1951) 354.
[13] C. A. VILLEE AND A. B. HASTINGS, *J. Biol. Chem.*, 179 (1949) 673.

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Enzymic synthesis and reduction of malonyl semialdehyde-coenzyme A

Studies on propionate metabolism in animal tissues have shown that malonate semialdehyde is formed by the oxidation of β-hydroxypropionate in the presence of DPN^+ and β-hydroxypropionic dehydrogenase[1,2] and by the enzymic transamination of β-alanine with α-ketoglutarate[3,4]. Malonate semialdehyde has also been implicated as an intermediate in propionate oxidation by peanut mitochondria[5]. More recently Vagelos and Earl[6] have demonstrated that extracts of *Clostridium kluyveri* oxidize β-hydroxypropionyl-CoA to malonyl semialdehyde-CoA in the presence of TPN^+.

The fact that the purified pig-heart succinyl–acetoacetyl-CoA transferase[7] was recently shown to catalyze the transfer of CoA from acetoacetyl-CoA or succinyl-CoA to malonate with formation of malonyl-CoA[8] suggested that malonate semialdehyde might also be a substrate for this enzyme. We wish to report that malonyl semialdehyde-CoA can indeed be synthesized enzymically by transfer of CoA from succinyl-CoA (or acetoacetyl-CoA) to the carboxyl group of malonate semialdehyde in the presence of CoA transferase according to the following reactions:

$$\text{Succinyl-CoA} + \text{malonate semialdehyde} \rightleftharpoons \text{malonyl semialdehyde-CoA} + \text{succinate} \qquad (1)$$

$$\text{Acetoacetyl-CoA} + \text{malonate semialdehyde} \rightleftharpoons \text{malonyl semialdehyde-CoA} + \text{acetoacetate} \qquad (2)$$

Reaction (1) was followed by a direct optical assay[7] based upon the absorption band (λ_{max} about 303 mμ) possessed by β-keto thioesters, including malonyl semialdehyde-CoA[6], in alkaline solution. When highly purified CoA transferase was added to succinyl-CoA and malonate semialdehyde at pH 8.0, a rapid increase in absorbancy at 310 mμ occurred (Fig. 1, lower curve), indicating the formation of malonyl semialdehyde-CoA. After the reaction had approached equilibrium, the pH of the reaction mixture was lowered by successive additions of dilute HCl and the changes in absorbancy followed. It is seen that the absorbancy of the reaction product decreases with decreasing pH, but unlike that of acetoacetyl-CoA[9] is appreciable at acid pH. This spectral behavior has been found for synthetic malonyl semialdehyde pantetheine[6] and is further evidence for the identity of the product as malonyl semialdehyde-CoA. The p*K* of the enol group of the latter compound is apparently considerably less than that of acetoacetyl-CoA, suggesting that at pH 8.0 the absorbancy of malonyl semialdehyde-CoA might be greater than that of acetoacetyl-CoA and that reaction (2) could, therefore, be measured optically. As shown in Fig. 1 (upper curve), the absorbancy of a solution of acetoacetyl-CoA at pH 8.0 decreases due to spontaneous deacylation. On addition of purified CoA transferase (in the presence of malonate semialdehyde), the absorbancy increases rapidly, thereby demonstrating the formation of malonyl semialdehyde-CoA.

Further evidence for the occurrence of reaction (1) was obtained by coupling it with reaction (3), which we have found to be catalyzed by β-hydroxybutyryl-CoA dehydrogenase, and demonstrating the net reaction (4) by direct optical test.

$$\text{Malonyl semialdehyde-CoA} + \text{DPNH} + H^+ \rightleftharpoons \beta\text{-hydroxypropionyl-CoA} + \text{DPN}^+ \qquad (3)$$

$$\text{Succinyl-CoA} + \text{malonate semialdehyde} + \text{DPNH} + H^+ \rightleftharpoons \beta\text{-hydroxypropionyl-CoA} + \text{DPN}^+ + \text{succinate} \qquad (4)$$

Abbreviations: CoA, coenzyme A; DPN^+, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN^+, TPNH, oxidized and reduced triphosphopyridine nucleotide.

Thus when malonate semialdehyde, DPNH, crystalline pig heart β-hydroxy-butyryl-CoA dehydrogenase[10], and CoA transferase were mixed, no reaction occurred (Fig. 2) as measured by the absorbancy of DPNH at 340 mμ. It is therefore evident that these enzyme preparations were entirely free of β-hydroxypropionic dehydrogenase. However, upon addition of succinyl-CoA, oxidation of DPNH was observed (Fig. 2, lower curve) as a result of synthesis and reduction of malonyl semialdehyde-CoA. As already shown with acetoacetyl-CoA[10], TPNH can replace DPNH in reaction 3 (Fig. 2, upper curve). This reduction of malonyl semialdehyde-CoA by a crystalline preparation of β-hydroxybutyryl-CoA dehydrogenase apparently explains the disappearance of malonyl semialdehyde-CoA catalyzed by crude extracts of pig heart and rabbit liver in the presence of DPNH (see ref. 6).

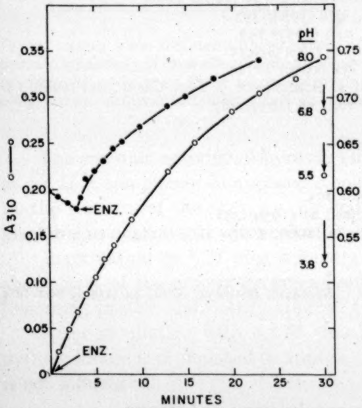

Fig. 1. The reaction mixture initially contained sodium pyrophosphate, 200 μmoles; malonic semialdehyde, 10 μmoles; and acetoacetyl-CoA, 0.27 μmoles (upper curve) or succinyl-CoA, 1.0 μmole (lower curve). Purified pig heart CoA transferase (3.2 μg; specific activity, 1000) was added at zero time (lower curve) and after 3 min (upper curve), to start the reaction. The final pH was 8.0. Volume, 1.50 ml, $d = 1.0$ cm, temperature, 22°. In the experiment represented by the lower curve after 30 min acid was added in increments to achieve the pH values shown and the resultant A_{310} values are indicated by the open circles. Thioester was omitted from the reference cuvette.[2]

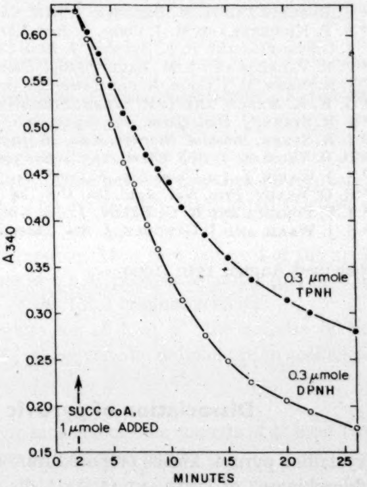

Fig. 2. The reaction mixture initially contained potassium phosphate buffer, pH 7.5, 100 μmoles; malonic semialdehyde, 10 μmoles; TPNH (upper curve) or DPNH (lower curve), 0.3 μmole; highly purified pig heart CoA transferase (8 μg; specific activity, 1000); and recrystallized pig heart β-hydroxybutyryl-CoA dehydrogenase (11.6 μg; specific activity, 149). At zero time the reaction was started by addition of 1 μmole succinyl-CoA to the experimental cuvette. Volume, 1.5 ml, $d = 1.0$ cm, temperature, 22°.

The evidence presented establishes an enzymic route for the conversion of malonic semialdehyde to its CoA ester and thereby provides additional support for the occurrence in animal tissues of a metabolic pathway for the β-oxidation of propionate. The function of such a pathway (other than in the formation of β-alanine) is not yet clear, particularly since the oxidation of malonyl semialdehyde-CoA to malonyl-CoA, although shown in a bacterial enzyme preparation[11], has not yet been demonstrated in animal enzyme preparations. It may be noted that the β-oxidation of propionate to malonyl-CoA would provide a means for the formation of the latter compound, an intermediate in fatty acid synthesis, other than by acetyl-CoA carboxylation[12-15].

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[1] G. Rendina and M. J. Coon, *J. Biol. Chem.*, 225 (1957) 523.
[2] H. Den, W. G. Robinson and M. J. Coon, *J. Biol. Chem.*, 234 (1959) 1666.
[3] E. Roberts and H. M. Bregoff, *J. Biol. Chem.*, 201 (1953) 393.
[4] F. P. Kupiecki and M. J. Coon, *J. Biol. Chem.*, 229 (1957) 743.
[5] J. Giovanelli and P. K. Stumpf, *J. Biol. Chem.*, 231 (1958) 411.
[6] P. R. Vagelos and J. M. Earl, *J. Biol. Chem.*, 234 (1959) 2272.
[7] J. R. Stern, M. J. Coon, A. del Campillo and M. C. Schneider, *J. Biol. Chem.*, 221 (1956) 15.
[8] G. K. K. Menon, and J. R. Stern, *Federation Proc.*, 18 (1959) 287.
[9] J. R. Stern, *J. Biol. Chem.*, 221 (1955) 33.
[10] J. R. Stern, *Biochim. Biophys. Acta*, 26 (1957) 448.
[11] P. R. Vagelos, *J. Biol. Chem.*, 235 (1960) 346.
[12] S. J. Wakil, *J. Am. Chem. Soc.*, 80 (1958) 6465.
[13] R. O. Brady, *Proc. Nat. Acad. Sci. U.S.*, 44 (1958) 993.
[14] J. V. Formica and R. O. Brady, *J. Am. Chem. Soc.*, 81 (1959) 752.
[15] S. J. Wakil and J. Ganguly, *J. Am. Chem. Soc.*, 81 (1960) 2597.

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Dissociation of pyruvic kinase in urea solutions

Crystalline pyruvic kinase prepared from rabbit muscle according to the method for "fluorokinase" of Tietz and Ochoa[1], dissociates in concentrated urea solutions into smaller fragments. In 2.5–3 *M* urea both forms (undissociated and dissociation products) are clearly separated in the ultracentrifuge (Fig. 1).

The sedimentation coefficient ($S_{20,w}$) of the undissociated form drops from 10.4 S in phosphate buffer (pH 6), to 7.5 S in 3 *M* urea, as a result of an increase in the friction coefficient. In 6 *M* urea the dissociation is complete, and the product sediments in the ultracentrifuge as a single, fairly symmetrical peak (Fig. 2). Its sedimentation coefficient, determined at 0.6% protein concentration in 6 *M* urea (dissolved in phosphate buffer, pH 6, I 0.1, for the elimination of charge effects) amounts to 2.2 S.

The diffusion coefficient ($D_{20,w}$) determined under the same conditions is $1.4 \cdot 10^{-7}$ cm^2/sec.

The molecular weight of the dissociation product calculated from the above values is 150,000, assuming the partial specific volume to be the same as for the intact enzyme, viz. 0.75 cm^3/g[2]. The friction coefficient (f/f_0) is 4.2. Native pyruvic kinase has a molecular weight within the range 230,000–237,000[2,3], so it seems that its molecule is split by strong urea into two parts. Since molecular-weight determinations in concentrated urea are uncertain[4], one cannot exclude the possibility that these fragments may be the halves of the original molecule.

Similar dissociation was observed at pH 12 in glycine buffer, but the dissociation products showed marked inhomogeneity in the ultracentrifuge.

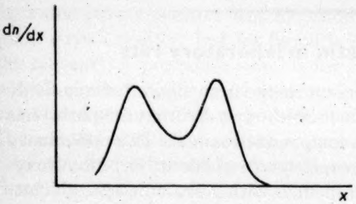

Fig. 1. Lamm scale sedimentation diagram of pyruvic kinase in 3 *M* urea after 60 min at 50,000 rev./min (184,000 × *g*). Protein concentration, 0.6 g/100 ml. Sedimentation from left to right.

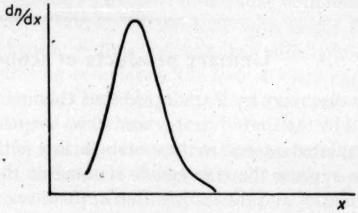

Fig. 2. Lamm scale sedimentation diagram of pyruvic kinase in 6 *M* urea (dissolved in phosphate buffer, pH 6, I, 0.1) after 180 min at 50,000 rev./min. Protein concentration, 0.6 g/100 ml.

The enzymic activity of pyruvic kinase diminishes in the presence of urea, and at 2.5 *M* it completely disappears. About 80 % of the activity can then be recovered by the removal of the urea, *e.g.* by dialysis. The irreversibly inactivated fraction is insoluble in neutral salt solutions of I, 0.1.

Inactivation by 5 *M* urea is mostly irreversible. After slow removal of the urea, about 80 % of the protein becomes insoluble in 0.1 *M* phosphate buffer (pH 6). The remaining protein shows only approx. 5 % of the total original activity.

After incubation with 0.1 *M* thioglycolate for 48 h at 2°, the activity of the pyruvic kinase is diminished to approx. 15 %. However, the sedimentation coefficient is not influenced.

These experiments show that the pyruvic kinase molecule consists of at least two polypeptide chains joined together by means of hydrogen bonds.

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[1] A. TIETZ AND S. OCHOA, *Arch. Biochem. Biophys.*, 78 (1958) 477.
[2] R. C. WARNER, *Arch. Biochem. Biophys.*, 78 (1958) 494.
[3] A. MORAWIECKI, *Acta Biochim. Polon.*, 5 (1958) 437.
[4] K. SCHACHMAN, *Ultracentrifugation in Biochemistry*, Academic Press, New York, 1959, p. 82.

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Preliminary Notes

Urinary products of scopoletin in laboratory rats

The discovery by YANG *et al.*[1] that the mainstream smoke from cigarettes commonly used in the United States contained scopoletin (6-methoxy-7-hydroxycoumarin) has stimulated interest in the metabolic fate of this compound in animals. This preliminary note reports the conversion of some of the scopoletin to esculetin (6,7-dihydroxycoumarin) and the conjugation of these two compounds with glucuronic acid, glycine, and sulfate in the rat. The conversion of scopoletin to esculetin is particularly interesting because a demethylation reaction is indicated.

The urine from three female albino rats (Sprague-Dawley), weighing 250–300 g each and fed a vitamin B complex test diet with complete vitamin fortification (Nutritional Biochemicals Corp., Cleveland, Ohio) for 3 weeks, was collected under toluene and spotted on S & S No. 589 red-ribbon chromatography paper (11½ in × 12 in). Each chromatogram was developed in the first direction in chloroform–acetic acid–water (2:1:1, v/v/v, lower layer) and then in the second direction in 20 % KCl–water. When observed under long-wavelength ultraviolet light (3660 Å), none of the compounds described below was found on the paper chromatograms. Then the same animals were fed the complete diet as previously mentioned, with the addition, however, of scopoletin (1 %). Upon analysis in like manner as the control, paper chromatograms of the urine collected after administration of the latter diet now contained at least three blue-fluorescing zones which were not found on the chromatograms of urine from the animals before they were fed scopoletin.

The separation of each of the individual fluorescent compounds was undertaken by chromatography on 7½ in × 21 in papers, using *n*-butanol–pyridine–benzene–water (5:3:1:3, v/v/v/v upper layer) as the solvent system. In this solvent system, using only one-dimensional chromatography, three major fluorescent zones, with R_F values of approx. 0.15, 0.55, and 0.89, were found on the chromatograms. Two other zones, with R_F values of 0.20 and 0.68, were present, but exhibited no fluorescence.

The farthest moving zone (R_F, 0.89) in this *n*-butanol–pyridine–benzene–water system was purified further by cutting the zone out of twelve such chromatograms, sewing each onto a new sheet of paper and developing them in *n*-butanol–acetic acid–water (6:1:2, v/v/v). The resulting major zone in each (R_F, 0.92) was cut out, eluted with 75 % ethanol–water, and the combined eluates were reduced in volume *in vacuo*. On comparison by paper chromatography in all the solvent systems mentioned in this note and by ultraviolet-absorption spectrophotometry, the purified compound in the concentrate was found to be identical with the reference scopoletin synthesized by BRAYMER *et al.*[2].

The zone with R_F 0.68 in *n*-butanol–pyridine–benzene–water was not visible under the ultraviolet light before spraying, but it produced a red color when sprayed with a diazotized sulfanilic acid solution[3]. Identification studies on this compound are now in progress.

The third zone (R_F, 0.55 in the *n*-butanol–pyridine–benzene–water) from each chromatogram was cut out, sewed onto new sheets of paper, and developed a second time in the same solvent system. After spraying, first with 3 *N* HCl, and secondly with a solution of 100 mg $BaCl_2$ in 50 ml 70 % ethanol, followed by a solution of sodium rhodizonate (10 mg in 20 ml of 50 % ethanol)[4], the part of the zone with lesser R_F value gave a positive test for sulfate. That portion of the zone with larger R_F value gave a positive test for hippuric acid using *p*-dimethylaminobenzaldehyde as the reagent[5]. A procedure suitable for separating completely the two or more compounds present in this third zone was not found. Therefore, the entire zone was cut out, eluted with 75 % ethanol–water, concentrated, and then hydrolyzed with 6 *N* HCl. The concentrated ethyl acetate extract of the hydrolysate contained scopoletin and esculetin, as determined by comparison with authentic samples of each, using paper chromatography in all the solvent systems already listed and also in 15 % acetic acid–water. The correct linkage and combinations of esculetin and scopoletin with the glycine and sulfate have not yet been established.

The combined eluates in 50 % methanol of the slowest moving zone (R_F, 0.15) from twenty chromatograms developed in the *n*-butanol–pyridine–benzene–water were evaporated to dryness *in vacuo*, and then dissolved in 25 ml phosphate buffer, pH 6. The solution was then incubated at 37° for 24 h with 50 mg β-glucuronidase, and then extracted with ethyl acetate. The ethyl acetate fraction contained mainly scopoletin, but a relatively much smaller amount of esculetin was also found by paper chromatographic analysis. Another aliquot of the eluate from this zone which was treated identically except that no enzyme was added contained neither free scopoletin nor esculetin. Thus, this zone contained mainly scopoletin glucuronide, but a relatively small amount of a glucuronide of esculetin also was present.

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[1] C. H. YANG, Y. NAKAGAWA AND S. H. WENDER, *J. Org. Chem.*, 23 (1958) 204.
[2] H. D. BRAYMER, M. R. SHETLAR AND S. H. WENDER, *Biochim. Biophys. Acta*, in the press.
[3] B. N. AMES AND H. K. MITCHELL, *J. Am. Chem. Soc.*, 74 (1952) 252.
[4] D. P. BURMA, *Anal. Chem.*, 25 (1953) 549.
[5] G. W. GAFFNY, *J. Biol. Chem.*, 206 (1954) 695.

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Etudes preliminaires sur la structure d'une α_1-glycoproteine acide (pleuromucoide)

On sait que les parties polypeptidique et glucidique des glycoprotéines sont unies par des liaisons de covalence, mais on ignore la nature de cette union et la séquence des unités glucidiques. POPENOE ET DREW[1] ont établi la position terminale de l'acide sialique; WINZLER[2] a étudié, par chromatographie qualitative sur papier, la libération des glucides au cours de l'hydrolyse acide de l'orosomucoide. Enfin SCHMID *et al.*[3] ont obtenu par clivage de cette protéine avec HCl gazeux à 0°, une fraction qui conserve la partie polypeptidique dans son état naturel, mais contient moins de la moitié du taux initial des glucides.

Le but de ce travail est d'obtenir, à partir d'une α_1-glycoprotéine, un ou plusieurs glycopeptides riches en glucides. Ce résultat est atteint par l'action d'enzymes protéolytiques, lesquels, contrairement aux assertions de YAMASHIMA[3], dégradent la glycoprotéine.

Ces enzymes sont utilisés dans la zone de leur pH d'activité maximum, à 37° et pendant un temps d'incubation de 10–16 h. Les hydrolysats enzymatiques sont ensuite additionnés de 10 vol. d'éthanol refroidi; le précipité formé est recueilli et le surnageant est concentré sous vide pour éliminer l'éthanol. Les déterminations des hexoses, hexosamines, 6-désoxyhexoses et acide sialique sont effectuées sur le précipité et sur le surnageant.

L'α_1-glycoprotéine utilisée est isolée du liquide pleural; son homogénéité est démontrée[5] et sa concentration en glucides est de 41 %; ses propriétés sont voisines de celles de l'orosomucoide.

La pepsine à pH 2, pendant 16 h, dégrade la glycoprotéine: le précipité éthanolique de l'hydrolysat correspond en poids à 70 % de la protéine originelle. Ce précipité contient la totalité des hexoses et des hexosamines, mais seulement 50 % des aminoacides et 50–60 % de l'acide sialique du produit initial; on retrouve 40–50 % d'acide sialique dans le surnageant, sous forme libre. En fait, la libération de cet acide n'est pas due à l'action hydrolysante de la pepsine mais à l'acidité du milieu. Ainsi, la fraction glycopeptide résultant de l'hydrolyse pepsique contient 22 % d'hexoses, 16.5 % d'acétylhexosamine, 2 % de fucose et 9 % d'acide sialique, soit un total de 50 % de substances glucidiques.

L'action de la papaine et de la chymotrypsine est plus faible, car on retrouve dans le précipité éthanolique, respectivement 90 et 80 % du substrat mis en jeu, avec la totalité des glucides de ce dernier. Le surnageant contient seulement des aminoacides et des peptides. La concentration en glucides du précipité éthanolique est de 45 %, dont 18 % d'hexoses et 11 % d'acide sialique dans le cas de la papaine; elle est de 50 % dont 20 % d'hexoses et 13.4 % d'acide sialique dans le cas de la chymotrypsine.

Enfin la trypsine s'est révélée l'enzyme la plus efficace, car elle libère 60 % d'aminoacides tandis que la totalité des glucides de l'α_1-glycoprotéine se retrouve dans le précipité éthanolique de l'hydrolysat trypsique. Le glycopeptide obtenu contient 24 % d'hexoses, 20 % d'acétylhexosamine, 3 % de fucose et 16 % d'acide sialique; la partie polysaccharidique de cette fraction représente 63 % de la molécule.

L'électrophorèse sur papier à pH 5 des différents précipités éthanoliques, met en évidence plusieurs bandes révélables par la ninhydrine; une seule bande est révélable par l'amidoschwarz et par le réactif de Schiff–acide périodique, sauf dans le cas de l'hydrolyse par la chymotrypsine où l'on observe 3 bandes colorées par ce dernier réactif.

Ces résultats nous ont conduit à conjuguer l'action de plusieurs enzymes pour tenter d'accroître encore le taux en glucides.

Après action de la trypsine sur l'hydrolysat pepsique de l'α_1-glycoprotéine, le précipité éthanolique représente seulement 50–55 % de la protéine mise en jeu et contient la totalité des hexoses et hexosamines initialement présents dans cette dernière. La concentration en glucides de ces glycopeptides représente 66 % de la molécule avec 30 % d'hexoses, 25 % d'acétylhexosamine, 8.3 % d'acide sialique et 2.6 % de fucose.

Enfin après action successive de la trypsine et de la papaine, puis électrophorèse de zone sur amidon de cet hydrolysat, on obtient un glycopeptide dont la concentration en glucides atteint 87 % avec 33.5 % d'hexoses, 28.8 % d'acétylhexosamine, 22 % d'acide sialique et 3.3 % de fucose. Ce glycopeptide contient la quasi-totalité des substances glucidiques de l'α_1-glycoprotéine originale.

Le Tableau I résume les modifications subies par l'α_1-glycoprotéine au cours des différentes hydrolyses protéolytiques. Il met l'accent sur le rôle capital de la trypsine dans la formation des glycopeptides.

TABLEAU I

POURCENTAGE EN GLUCIDES, DANS LE PRÉCIPITÉ ÉTHANOLIQUE OBTENU
À PARTIR DES DIFFÉRENTS HYDROLYSATS ENZYMATIQUES

Enzyme	*Hexosamines*	*Acetylhexosamines*	*6-Desoxyhexoses*	*Acide sialique*	*Totale en glucides*
	%	%	%	%	%
Néant	15.8	13.25	2	10.4	41.5
Pepsine	21.7	16.5	2.1	9.05	50
Papaine	18.2	—	—	10.95	45
Chymotrypsine	20.2	—	—	13.4	50
Trypsine	24.1	20	3	16	63
Pepsine + trypsine	30	25	2.6	8.3	66
Trypsine + papaine + électrophorèse sur amidon	33.5	28.8	3.3	22	87.5

Cette note préliminaire montre que les enzymes protéolytiques dégradent la glycoprotéine α_1 et qu'ils peuvent être utilisés dans l'étude de la structure de cette protéine. L'investigation sur la chimie des glycopeptides ainsi obtenus est en cours.

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[1] E. A. POPENOE ET R. M. DREW, *J. Biol. Chem.*, 228 (1957) 673.
[2] R. J. WINZLER, in *Ciba Foundation Symposium on the Chemistry of mucopolysaccharides*, 1958, p. 245.
[3] K. SCHMID, W. L. BENCZE, T. NUSSBAUMER ET J. O. WEHRMULLER, *J. Biol. Chem.*, 234 (1959) 529.
[4] I. YAMASHIMA, *Acta Chim. Scand.*, 10 (1956) 1066.
[5] R. BOURRILLON, J. MICHON ET R. GOT, sous presse.

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The effect of dimethylformamide on phosphoglyceraldehyde dehydrogenase

In earlier communications we have reported that the steric structure of phosphoglyceraldehyde dehydrogenase is comparatively labile. Relative small alterations in the steric structure (changes in the state of H-bonds, of sulfhydryl and polar groups, displacement of the firmly bound coenzyme) leads to striking changes in its enzymic activity as well as in its structure[1,2]. We believe that the reason for this lability is that the enzyme contains no disulfide linkages[3], which are particulary important for the stabilisation of the steric structure of other protein molecules.

The present paper reports some data on the importance of the apolar amino acid side chains for the steric structure and biological activity. According to YANG AND DOTY[4] and BRESSLER *et al.*[5] the water-miscible N,N′-dimethyl formamide is able to change the conformation of proteins by solvation of their apolar amino acid residues. Changes in the enzymic activity as well as in the optical rotation and viscosity of pig-muscle phosphoglyceraldehyde dehydrogenase by treatment with dimethylformamide have been studied.

(1) Physico-chemical measurements (Table I) revealed that progressive solvation of the apolar groups results mainly in swelling of the protein, which causes an increase in the hydrodynamic volume. The changes in the viscosity are more pronounced than those in the optical rotation. 25 % or more dimethylformamide suddenly and irreversibly denatures the protein.

TABLE I

THE EFFECT OF DIMETHYLFORMAMIDE ON THE SPECIFIC OPTICAL ROTATION AND VISCOSITY OF PIG-MUSCLE PHOSPHOGLYCERALDEHYDE DEHYDROGENASE

Dimethylformamide (%, v/v)	*Specific optical rotation* $[\alpha]^{20}_{D}$		*Intrinsic viscosity** $[\eta] \times 10^2$, *dl/g*	
	Time of preincubation (min)		*Buffers*	
	10	*30*	*0.2 M phosphate, pH 6.5*	*0.2 M glycine, pH 8.4*
0	— 31.3	— 31,3	2.95	3.35
5	—	—	3.45	4.28
10	— 32.8	— 33.0	5.10	5.90
15	— 36.0	— 36.9	6.40	7.95
20	— 38.4	—	7.55	6.75

* Time of preincubation 15 min. The data are averages of measurements carried out between 15 to 40 min after addition of dimethylformamide.

In contrast to serum albumin[5] the swelling and unfolding of phosphoglyceraldehyde dehydrogenase following apolar solvation of the hydrophobic groups causes a rapid and irreversible breakdown of its steric structure. Serum albumin when treated with hydrophobic solvents responds by "accommodation", *i.e.* the molecule turns with its hydrophobic amino acid residues towards the solvent.

(2) Two phases can be distinguished in the effect of DMF on the enzymic activity (Fig. 1):

Abbreviations: DPN, diphosphopyridine nucleotide.

(a) When dimethylformamide is added immediately before determination of the activity by Warburg's optical method the velocity of DPN reduction increases with increasing dimethylformamide concentration up to 15 % (v/v). At this point the activity is about 50 % higher than that of the control sample. Further increases in concentration of dimethylformamide (from 15 to 25 %) causes a decrease of the activity to the original value.

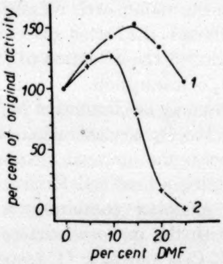

Fig. 1. The effect of dimethylformamide on the enzymic activity of pig- muscle phosphoglyceraldehyde dehydrogenase. Reaction mixture: D-glyceraldehyde 3-phosphate 0.1 μmole; DPN, 0.08 μmoles; arsenate 0.1 μmole; phosphoglyceraldehyde dehydrogenase, $2 \cdot 10^{-5}$ μmole; cysteine 0.5 μmole; dimethylformamide (DMF) and 0.2 *M* glycine buffer up to an and volume of 3.0 ml. Final pH 8.4. Curve 1, no preincubation of enzyme and dimethylformamide; curve 2, pre-incubation for 60 min at room temperature.

(b) When the enzyme is preincubated with dimethylformamide at room temperature for 30 to 60 min, activation is observed only up to about 10 % dimethylformamide. Treatment for 60 min with 25 % dimethylformamide causes almost complete inactivation.

We conclude that an aqueous medium may not represent the optimal conditions for maximal activity of phosphoglyceraldehyde dehydrogenase. It seems that up to a point, a small swelling of the protein molecule increases the catalytic function.

Detailed experimental data on the effect of dimethylformamide on phosphoglyceraldehyde dehydrogenase and other enzymes will be published elsewhere.

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[1] P. ELÖDI, *Biochim. Biophys. Acta*, 40 (1960) 272.
[2] P. ELÖDI AND GY. JÉCSAI, *Acta Physiol. Hung.*, 17 (1960) 175.
[3] P. D. BOYER AND A. R. SCHULZ, *Symposium on Sulfur in Proteins*, Academic Press, New York, 1959, p. 199.
[4] J. T. YANG AND P. DOTY, *J. Am. Chem. Soc.*, 79 (1957) 761.
[5] S. E. BRESSLER, V. P. KUSHNER AND S. YA. FRENKEL, *Biokhimiya*, 24 (1959) 685.

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Oxygen consumption of the frog gastric mucosa

The gastric mucosa of the frog *in vitro* maintains an electrical potential difference and secretes HCl for many hours. Both electrical and secretory activity are strongly dependent on respiration, disappearing quickly after removal of external oxygen[1]. The molar ratio of HCl secreted to O_2 consumed by the mucosa has been used to evaluate various proposed mechanisms for the production of acid[2]. Previous estimates of the ratio[3,4] have been based on the manometric measurement of O_2 consumption in the absence of external CO_2, although the latter gas is necessary for maximal acid secretion[5]. This method also precluded the detection of changes in electrical activity and any associated changes in O_2 consumption.

These experimental difficulties may be eliminated by the use of the polarographic method for the measurement of the O_2 concentration in the solutions bathing the isolated mucosa. In our experiments the mucosal membrane was mounted between two Lucite chambers, one containing a modified Krebs solution in contact with the serosal surface and the other chamber containing a similar solution, without bicarbonate buffer, in contact with the mucosal surface. These solutions had been equilibrated with 95 % O_2 + 5 % CO_2 and pure O_2 respectively. The concentration of O_2 in each solution was measured by an O_2 electrode[6] and always exceeded 50 % saturation. An electrical circuit permitted the continuous measurement of the current delivered by the mucosa into an external resistance of zero, *i.e.*, the short-circuit current. Acid secretion was measured by electrometric titration *in situ*. The solutions were stirred magnetically and were closed to the atmosphere. Measurements of the rates of acid secretion and O_2 removal from the two solutions were made for two to four successive intervals of 50 to 60 min each.

It was found that the rates of removal of O_2 from the two solutions were comparable; these two values have been added to find the total rate of O_2 consumption. All results have been expressed in µmoles or µequiv./mg dry wt. of mucosa/h. Table I lists the means, with corresponding standard errors, for 42 periods during which the mucosae were secreting spontaneously, and for 15 periods during which the mucosae were stimulated by $5 \cdot 10^{-5}$ *M* histamine.

TABLE I

	Acid secretion (µequiv./mg/h)	*Short-circuit current (µequiv./mg/h)*	*O_2 consumption (µmoles/mg/h)*
Spontaneous (42 periods)	0.079	0.362	0.139
S.E.*	± 0.010	± 0.023	± 0.011
Stimulated (15 periods)	0.150	0.360	0.168
S.E.*	± 0.021	± 0.027	± 0.015

* Standard error.

As found previously[7], the short-circuit current in these experiments does not depend on the rate of acid secretion. This finding makes possible an estimate of the ratio $\Delta H/\Delta O_2$ from Table I. Using the means of acid secretion and O_2 consumption, $\Delta H/\Delta O_2 = 0.071/0.029 = 2.4$.

In an alternate analysis, it was assumed that the data could be fitted to an equation of the form:

$$\text{Oxygen consumption} = A + k_1\,(\text{acid secretion}) + k_2\,(\text{short-circuit current})$$

The constant A represents the O_2 consumed in processes other than those responsible for current or acid production. The values for k_1, k_2 and A were obtained by a conventional, least-squares technique[8]. A preliminary calculation showed that the use of histamine did not significantly affect the value of A, and the 57 experimental periods were combined to yield the values:

$$\begin{array}{ll} k_1 & 0.41 \pm 0.08 \text{ (standard deviation)} \\ k_2 & 0.27 \pm 0.04 \\ A & 0.01 \pm 0.05 \end{array}$$

By the use of the t test, in which $t_1 = 0.41/0.08 = 5.1$ and $t = 0.27/0.04 = 6.7$, we may virtually exclude the possibility that the O_2 consumption is independent of acid secretion ($k_1 = 0$) or short-circuit current ($k_2 = 0$). The value obtained for A indicates that nearly all the respiration is related to secretory and electrical activity.

The reciprocal of k_1, $1/0.41 = 2.4$, gives $\Delta H/\Delta O_2$ at constant short-circuit current; the reciprocal of k_2, $1/0.27 = 3.7$, yields Δ (short-circuit current)/ΔO_2 at constant acid secretion. CRANE AND DAVIES[3] found for the ratio $\Delta H/\Delta O_2$ values up to 13, with only one-third of the measurements yielding a value less than 4. DAVENPORT[4] has, however, reported a value of 2.15 for this ratio. The present results (and those of DAVENPORT) are consistent with the hypothesis that secreted H^+ is derived by a "redox" mechanism from substrate hydrogen (*cf.* ref. 9). A similar mechanism, presumed separate[10], may give rise to the transport of Cl^- manifested by the electrical current. These results leave open the question of linkage between the two mechanisms.

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[1] E. E. CRANE, R. E. DAVIES AND N. M. LONGMUIR, *Biochem. J.*, 43 (1948) 321.
[2] E. HEINZ AND K. J. ÖBRINK, *Physiol. Rev.*, 34 (1954) 643.
[3] E. E. CRANE AND R. E. DAVIES, *Biochem. J.*, 49 (1951) 169.
[4] H. W. DAVENPORT, *Fed. Proc.*, 11 (1952) 715.
[5] C. A. M. HOGBEN, in A. M. SHANES, *Electrolytes in Biological Systems*, American Physiological Society, Washington, 1955.
[6] L. C. CLARK, JR., *Trans. Am. Soc. for Int. Organs*, 11 (1956) 41.
[7] R. P. DURBIN AND E. HEINZ, *J. Gen. Physiol.*, 41 (1958) 1035.
[8] G. W. SNEDECOR, *Statistical Methods*, Iowa State College Press, Ames, 1957.
[9] E. J. CONWAY, in *The Method of Isotopic Tracers Applied to the Study of Active Ion Transport (Saclay)*, Pergamon Press, New York, 1959.
[10] E. HEINZ AND R. P. DURBIN, *Biochim. Biophys. Acta*, 31 (1959) 246.

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